IMMUNE MECHANISMS GOVERNING INFECTION CONTROL AND PATHOGENESIS OF *HELICOBACTER PYLORI* AND PROSPECTS FOR A HUMAN VACCINE

DISSERTATION

ZUR

ERLANGUNG DER NATURWISSENSCHAFTLICHEN DOKTORWÜRDE

(DR. SC. NAT.)

VORGELEGT DER

MATHEMATISCH-NATURWISSENSCHAFTLICHEN FAKULTÄT

DER

UNIVERSITÄT ZÜRICH

VON

IRIS HITZLER

AUS

DEUTSCHLAND

PROMOTIONSKOMITEE:

PROF. DR. ANNE MÜLLER (VORSITZ UND LEITUNG DER DISSERTATION)

PROF. DR. BURKHARD BECHER

PROF. DR. MARIES VAN DEN BROEK

PROF. DR. ROBERTO SPECK

ZÜRICH, 2011

ZUSAMMENFASSUNG

Infektion mit dem Gram-negativen Bakterium *Helicobacter pylori* ist weitverbreitet und der hauptsächliche Entstehungsgrund von Magen- und Zwölffingerdarmgeschwüren, des Adenokarzinoms des Magens und des MALT-Lymphoms. Die gegenwärtige Standardbehandlung ist Eradikationstherapie, jedoch nimmt die Antibiotikaresistenz zu und in manchen Regionen sind die Reinfektionsraten hoch. Da ausserdem die meisten Patienten keine Symptome zeigen bevor Magenkrebs diagnostiziert wird, wäre ein Impfstoff von grosser Hilfe und würde eine kosteneffiziente Handhabung der Infektion ermöglichen. Diverse Strategien wurden in Tiermodellen und auch im Menschen getestet, allerdings wurde bisher keine wirksame und sichere Vakzine etabliert. Wir haben uns daran gemacht die Mechanismen zu bestimmen, die im erfolgreichsten Impfmodell am Werk sind, um mit diesen Erkenntnissen anschliessend einen neuen Ansatz für die menschliche Impfung zu definieren. Wir konnten zeigen, dass die Reduzierung der Bakterienzahl von $CD4^+$ T-Helferzellen der Th1- und Th17-Typen abhängt, sowie von Neutrophilen und, zu vermutlich geringerem Ausmass, von Mastzellen. Mit CAF01 haben wir ein Adjuvans identifiziert das für den menschlichen Gebrauch unbedenklich wäre und die gewünschte Art von Immunantworten auslöst. Die Herausforderung sterile Immunität zu erreichen bleibt jedoch bestehen. Wir konnten darlegen dass Dendritische Zellen, die durch Kontakt mit *H. pylori* tolerogen werden, einen wichtigen Mechanismus darstellen, der die *H. pylori*-spezifische Immunität herunterreguliert und überwunden werden muss. Gleichzeitig gibt es zunehmende Belege dass die durch *H. pylori* hervorgerufene Immunantwort wesentlich zur Pathologie von infizierten Personen beiträgt. Nachdem wir zeigen konnten dass Th1-Antworten, welche durch normale Infektion und erst recht durch Impfung ausgelöst werden, präneoplastische Läsionen der Magenmukosa vorantreiben, widmeten wir uns der Frage, ob suboptimale Impfung die Entstehung von Gastritis und Pathologie eher beschleunigen als verhindern könnte. Zu verschiedenen Zeitpunkten nach Infektion beobachteten wir tatsächlich verstärkte pathologische Veränderungen in immunisierten Mäusen, die nicht in der Lage waren die Infektion komplett zu eliminieren, im Vergleich zu naiven Kontrollen. Dies unterstreicht dass jeder Impfstoff, der keine sterile Immunität schafft, das Magenkrebsrisiko des Geimpften wahrscheinlich erhöht. Abgesehen davon konnten wir neue Einblicke in *H. pylori*'s Interaktion mit dem Immunsystem gewinnen, indem wir das Inflammasom mit seinem Hauptakteur Caspase-1 als einen entscheidenden Faktor für den Ausgang der Infektion herausgestellt haben. Durch die Prozessierung seiner Substrate IL-1β und IL-18 in Antwort auf Signale von *H. pylori*

trägt dieses Enzym zu *H. pylori*-spezifischer Immunität bei, reguliert sie aber gleichzeitig auch herunter. Die zukünftigen Herausforderungen werden darin bestehen, die gewonnenen Erkenntnisse zur Verbesserung der Impfstrategien zu nutzen und diese für das frühkindliche Alter anzupassen. Es sollte neu eingeschätzt werden ob Vakzinierung eine sinnvolle Strategie darstellt angesichts der möglichen Verstärkung der Immunpathologie und positiver Aspekte der Infektion mit *H. pylori* in Bezug auf andere Krankheiten.

SUMMARY

Infection with the Gram-negative bacterium *Helicobacter pylori* is widespread and the main etiologic cause of peptic ulcers, gastric adenocarcinoma and MALT lymphoma. The current treatment standard is eradication therapy, but antibiotic resistance is rising and in some areas reinfection rates are high. Since most patients do not display any symptoms before gastric cancer is diagnosed, a vaccine would be of great help and enable cost-effective infection management. Various strategies have been attempted in animal models and also human volunteers, yet an efficient and safe vaccine regimen has not been established so far. We set out to determine the mechanisms that are at work in the most successful animal model of vaccination to subsequently use these findings to design a new approach for human vaccination. We could show that reduction of bacterial loads depends upon $CD4^+$ helper T cells of the Th1 and Th17 types, as well as neutrophils and, presumably to a lesser extent, mast cells. With CAF01 we have identified an adjuvant that would be safe for human use and elicits the desired type of immune responses, however the challenge of achieving sterilizing immunity persists. We could point out dendritic cells rendered tolerogenic by contact with *H. pylori* as one important mechanism that is downregulating *H. pylori*-specific immunity and will need to be overcome.

At the same time, there is increasing evidence that the immune response elicited by *H. pylori* contributes substantially to the pathology observed in infected individuals. Having shown that Th1 responses, which are elicited by normal infection and even more so by vaccination, are driving preneoplastic lesions of the gastric mucosa, we addressed the question if suboptimal vaccination could enhance gastritis and pathology formation rather than preventing it. At different time points after challenge infection we indeed observed aggravated pathological changes in immunized/infected mice unable to completely clear the infection compared to naive controls. This underlines that every vaccine that fails to achieve sterile immunity likely increases the gastric cancer risk of the vaccinee. Apart from that, we have gained novel insights into *H. pylori*'s interaction with the immune system by identifying the inflammasome with its main player caspase-1 as a crucial determinant of infection outcome. By processing its substrates IL-1β and IL-18 in response to *H. pylori*-derived signals, this enzyme contributes to *H. pylori*-specific immunity but at the same time downregulates it. The future challenges will be to use the gained knowledge for improving vaccination strategies and adopt these to the neonatal period. It should be reappraised if vaccination is a valid strategy in light of potential aggravation of immunopathology and beneficial aspects of *H. pylori* infection regarding other diseases.

TABLE OF CONTENTS

ABBREVIATIONS

αGalCer	α-galactosylceramide
AlOH	aluminium hydroxide
BMDC	bone marrow derived dendritic cell
CAF01	cationic adjuvant formulation 01
CagA	cytotoxin-associated gene A
CD40L	CD40 ligand
CFA	Complete Freund's Adjuvant
CFU	colony forming unit
CpG-ODN	CpG oligodeoxynucleotides
CT	Cholera toxin
DAMP	damage-associated molecular pattern
DC	dendritic cell
IFN-γ	interferon gamma
Ig	immunoglobulin
IL-X(R)	interleukin X (receptor)
LPS	lipopolysaccharide
LT	*Escherichia coli* heat-labile toxin
MALT	mucosa-associated lymphoid tissue
MHCII	major histocompatibility complex class II
MPL	monophosphoryl lipid A
MPO	myeloperoxidase
NAP	neutrophil-activating protein
NK	natural killer
NLR	NOD-like receptor
PAMP	pathogen-associated molecular pattern
PMSS1	pre-mouse SS1
PPI	proton pump inhibitor
PRR	pattern recognition receptor
Rag-1	recombination activating gene 1
SAS	Sigma Adjuvant System®
SS1	Sydney strain 1
T4SS	type IV secretion system
TCR	T cell receptor
TGF-β	transforming growth factor beta
Th1/ 2 / 17	T helper type 1/ 2/ 17
TLR	Toll-like receptor
TNF-α	tumor necrosis factor alpha
Treg	regulatory T cell
UreA/ B	subunit A/ B of urease
VacA	vacuolating cytotoxin

1. INTRODUCTION

1.1 *Helicobacter pylori*

1.1.1 Taxonomy

The *Helicobacter* genus comprises Gram-negative bacteria with curved to spiral shape that are microaerophilic and highly motile by virtue of one or multiple flagella (see Fig. 1).[1] More than 30 species of *Helicobacter* have been identified so far.[2] The first one, *Helicobacter pylori*, was discovered in the human stomach and named after its primary location near the pylorus, the stomach's connection to the duodenum (see Fig. 2). Subsequently, *Helicobacter* species were also isolated from several animal species, including cats, dogs, monkeys, sheep, pigs, rodents, birds, cheetahs and poultry.[3]

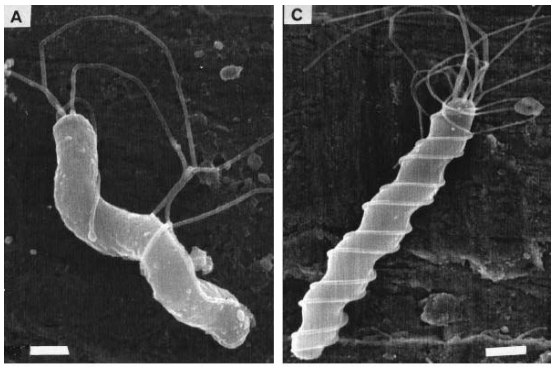


Fig. 1. Morphology of *Helicobacter* species. S-shaped *H. pylori* with five to seven sheathed polar flagella (left) and helical-shaped *H. felis* with periplasmic fibers in pairs and bipolar tufts of sheathed flagella (right). Field emission SEM, bar marker = 0.5 µm. From [1].

Helicobacter can be broadly grouped according to whether they colonize the gastrointestinal or hepatobiliary niche. They do not possess a reservoir outside their hosts. Species that can colonize the liver of mammals include *H. hepaticus* and *H. canis*. *H. felis* and *H. heilmanii* are examples of other *Helicobacter* found to colonize the stomach, in cats, dogs and primates, respectively.

1.1.2 Virulence factors

All *Helicobacter* species are characterized by high expression levels of urease, an enzyme which efficiently converts urea into carbonic acid and ammonia. The latter is believed to neutralize the periplasmic pH, providing the gastric colonizers with an acid tolerance that is critical to establish

stable colonization of their hosts.[4] Urease is therefore an essential virulence determinant of gastric *Helicobacter.*[5, 6] Motility is also a survival factor, since the bacteria must reach the safety of their niche, the gastric mucosa, where the pH is between 4 and 6.5, and otherwise only outlast some days.[7] *H. pylori* resides mostly within the mucus layer (85%), while 10% of the bacteria adhere to epithelial cells and 5% are found at intercellular junctions.[8, 9]

Several more factors determine *H. pylori*'s virulence. The Cytotoxin-associated gene A (CagA) protein, found in 50 to 70% of *H. pylori* strains,[10] is the main identified substrate of *H. pylori*'s type IV secretion system (T4SS) and as such translocated into host cells where it gets phosphorylated.[11, 12] The described functions of CagA are manifold. Besides inducing morphological changes in epithelial cells, it can elicit apoptosis of T cells.[10] The vacuolating cytotoxin VacA is secreted by about half of all *H. pylori* strains and has been attributed an inhibitory effect on T cells[13] and various effects on epithelium, especially pore formation.[14]

Moreover, *H. pylori* possesses a variety of adhesins, mediating its adhesion to epithelial cells, such as BabA. The surface-associated protein HpaA has an unclear role but is essential for colonization.[15] Also strains lacking neutrophil-activating protein (NAP) exhibit decreased survival compared to wild type.[16] The described roles of this protein include, as its name says, neutrophil recruitment, stimulation of host cell production of reactive oxygen intermediates, but at the same time combating oxidative stress within *H. pylori* and iron sequestration.[16, 17]

In general, *H. pylori* strains can be classified into two types: type I strains possess VacA and CagA, type II strains lack both virulence factors or at least their functionality. *H. pylori* demonstrates a huge heterogeneity, with differences concerning the level of virulence, for example due to the varying number of phosphorylation motifs present in CagA,[18] or other factors. Strain diversity is not only observed among different populations but even individuals seem to carry their own unique strain or several of them, owing to the bacteria's capability to continuously adapt to their environment by mutations and DNA rearrangements.[19]

1.1.3 Prevalence and transmission

In total, more than half of the global population is nowadays infected with *H. pylori.*[20] The prevalence reaches over 80% in parts of the developing world whereas in industrialized countries it is steadily decreasing and ranging from 20 to 50%.[21] Infection with this very successful human pathogen occurs mainly in infancy.[22, 23] Whilst the infection has been described to be of transient nature in some infants, i.e. spontaneous clearance can occur,[22, 24, 25]

once it is established, it persists for life unless treated. Final evidence concerning the mode of transmission is lacking. It is commonly believed to happen mainly through direct person-to-person contact within families, via the oral-oral route or also the fecal-oral route.[26] Additionally, transmission via contaminated food or water is regarded as one possible mechanism, at least in developing countries.[27] Generally, socioeconomic status has been shown to be linked with infection and the improvement of hygienic standards is believed to be the reason for the dramatic decrease of *H. pylori* prevalence in many areas.[26]

1.2 *H. pylori* and gastric cancer

1.2.1 Epidemiology

H. pylori is accepted today to be the main etiological factor for developing gastritis, gastric and duodenal peptic ulcers, and eventually gastric adenocarcinoma or mucosa-associated lymphoid tissue (MALT) lymphoma.[28] The initial proof of this causal relationship was delivered by Marshall and Warren in 1983[29, 30] and awarded the Nobel prize for Physiology and Medicine in 2005. The medical community had to abandon the hypothesis of stress being the primary factor causing gastric mucosal disease. Since this fundamental discovery, it has been established that *H. pylori* infection causes transient acute gastritis that in most cases turns into chronic active gastritis. The majority, namely about 80%, of infected individuals remain clinically asymptomatic despite life-long persistence of the infection. 10 to 15% will develop peptic ulcer disease, which can either affect the stomach or the duodenum, depending on the location of the gastritis more in the body or the antral area of the stomach (see Fig. 2, 3).[10, 28]

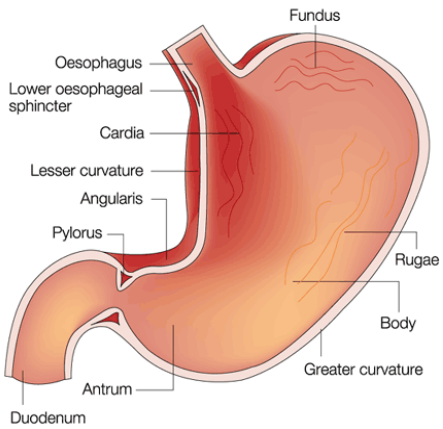


Fig. 2. Anatomical arrangement of the distal oesophagus, stomach and proximal duodenum. *H. pylori*-induced inflammation can occur at any site within the stomach. Most adenocarcinomas associated with *H. pylori* occur in the gastric antrum, body, or (less likely) fundus. From [31].

Gastric adenocarcinoma arises in 1 to 2% of infected individuals, and there is a risk of below 1% to develop MALT lymphoma.[10, 28, 32, 33] While ulcers can already occur in infected individuals around the age of 30, gastric cancer takes several more decades to develop (see Fig. 3).[8]

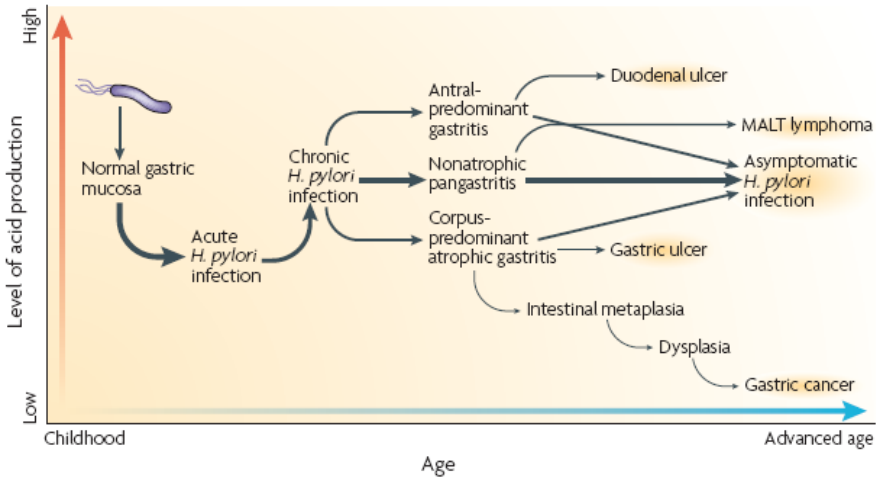


Fig. 3. Courses of *H. pylori* infection. From [19].

In light of this epidemiologic evidence, *H. pylori* has been the first and so far only bacterium classified as a class I carcinogen by the World Health Organization.[34] Given its high prevalence, *H. pylori* infection causes a considerable number of cancer cases, and stomach cancer, though decreasing in the Western world, remains the second most common cause of cancer-related death, having caused 740.000 deaths worldwide in 2008.[35]

1.2.2 Treatment

Patients that manifest with symptoms of peptic ulcer are routinely diagnosed for *H. pylori* status, mostly by histology or culture from gastric specimens obtained during endoscopy. The gold standard noninvasive method is urea breath testing for detection of urease activity.[10] If *H. pylori* is detected, the most commonly recommended treatment is the so-called triple therapy consisting of a proton pump inhibitor (PPI) combined with two antibiotics, clarithromycin and amoxicillin or metronidazole. The initial healing of ulcers is mainly achieved by the PPI, or alternatively a H2 blocker, both reducing the amount of gastric acid. The successful eradication of the infection only has a small benefit on the healing process but it dramatically reduces the risk of recurring gastric or duodenal ulcers.[36] However, several drawbacks come along such first-line treatment. First of all, it requires good patient compliance

since it is usually given during at least two weeks several times a day. Moreover, eradication therapy is nowadays only efficient in 75 to 80% of the cases, mainly due to increasing clarithromycin resistance.[37] And finally, even successful eradication does not definitely protect the patient: reinfection rates are quite high in some countries[38, 39] and, most notably, gastric cancer can develop even after cure of the infection, especially in cases where gastric mucosal atrophy has already proceeded to a certain stage at the time of treatment.[40] In the unfortunately frequent case that no symptoms manifest before gastric cancer is diagnosed, the only therapy consists in resection of the tumor. Additional eradication therapy apparently reduces to some extent the risk of recurrent gastric cancer.[41, 42] The possible implementation of a "test-and-treat" strategy, that is testing for and eradicating *H. pylori* in whole populations, is subject of much discussion. It has been suggested that such a strategy would reduce gastric cancer incidence and be cost-effective, at least in populations at high risk of developing gastric cancer.[43-45] However, so far no country, also not in high-risk regions, has adopted public health measures to treat *H. pylori* infection.[46]

1.2.3 Mechanisms of carcinogenesis

It is nowadays believed that the initiation of mucosal changes and progression toward gastric cancer is driven jointly by the bacteria, the environment and the host's predisposition.[47]

Concerning bacterial factors, it has been found that type I, i.e. $CagA^+$ and $VacA^+$, strains are generally more carcinogenic in humans[48] and in experimentally infected animals.[49] It has been suggested that CagA directly elicits several oncogenic mechanisms in host cells based on *in vitro* experiments. Upon injection via the T4SS and phosphorylation, CagA aberrantly activates the oncoprotein SHP-2 tyrosine phosphatase.[50] Independently of its phosphorylation, it causes junctional and polarity defects in epithelial cells[51] and leads to perturbed E-cadherin/beta-catenin signaling.[52] In a mouse model overexpressing CagA predominantly in the stomach, development of adenocarcinoma was observed,[53] however, the reported incidence was very low (<3 %). Moreover, bacterial strains lacking functional CagA are also causing ulcers and cancer in humans, albeit at a lower frequency.[10] The fact that weaker immune responses are induced and tumorigenesis is delayed, but not prevented in INS-GAS mice (see section 1.3) infected with a *H. pylori* strain that is unable to translocate CagA[54] points in the same direction. Therefore, the immediate effects of the bacteria on host cells have to be regarded as only one of several mechanisms of *in vivo* carcinogenesis.

Apart from that, various environmental influences have an impact on the outcome of *H. pylori* infection. A high salt diet, smoking, alcohol and use of proton pump inhibitors increase an infected individual's risk of developing gastric cancer, whereas use of non-steroidal anti-inflammatory drugs decreases it.[10, 55] A higher basal level of acid secretion and antral gastritis rather than corpus-predominant gastritis also predispose to a lower cancer risk.[8] Coinfection with a helminth parasite has been shown to dampen gastric pathology in C57BL/6 mice[56] and could explain the so-called "African enigma": in countries where a high prevalence of *H. pylori* is accompanied by widespread infection with gastrointestinal parasites, the gastric cancer incidence is unexpectedly low.

The third factor coming into play is the host's immune response. Polymorphisms in the gene encoding the proinflammatory cytokine interleukin (IL)-1β or its receptor, leading to enhanced signaling, have been shown to correlate with an increased risk of gastric cancer.[57] Constitutive stomach-specific overexpression of human IL-1β in a transgenic mouse model is sufficient to give rise to gastric cancer.[58] Further associations have been found for polymorphisms leading to low expression of IL-10[59] or increased expression of the interferon gamma (IFN-γ) receptor.[60, 61]

1.3 Animal models of *H. pylori* infection, pathogenesis and vaccination

In order to study details of *H. pylori* infection and the associated pathogenesis as well as the effects of treatment and vaccination, several animal models have been established, each with specific advantages and drawbacks. Which one is most appropriate depends on the experimental hypothesis. Mouse models are most widely used and especially valuable in light of the fact that various transgenic and knock-out strains are available. Since it was initially not possible to infect mice with virulent *H. pylori* isolates, except immunodeficient strains,[62] the first murine model established used the close *H. pylori* relative *H. felis*, initially isolated from cats.[63] However, *H. felis* differs considerably from *H. pylori* in that it possesses different virulence factors and lacks both CagA and VacA. Additionally it is hard to generate genetic mutants.[10] Therefore a lot of effort was made to adapt *H. pylori* to the mouse, which was finally achieved by passaging freshly isolated clinical isolates.[64] Lee et al. introduced the to date most widely used laboratory strain, mouse-adapted Sydney strain 1 (SS1),[65] which stably colonizes the murine stomach and harbors an unfunctional T4SS.[66] In addition, clinical isolates such as the

strain G27 are commonly used for *in vitro* studies with human cell lines. Our group recently described the use of the parental clinical isolate of SS1, pre-mouse SS1 (PMSS1),[67] which still has a functional T4SS, as an additional model which allows to study the role of the CagA translocation machinery *in vivo*.

Mouse models are useful for studying various *H. pylori* mutants in terms of their colonization behavior and host immune responses towards them. Moreover, the C57BL/6 strain develops gastritis and precancerous lesions after *H. felis* or *H. pylori* infection, which recapitulate the multistep process of cancer development observed in humans. These steps involve chronic inflammation (gastritis), progressive loss of specialized gastric cells (atrophy), accompanied by hyperproliferation of progenitor cells (hyperplasia) and/or appearance of intestinal goblet cell-like cells (intestinal metaplasia), and finally dysplasia (see Fig. 4). However, progression to malignant carcinoma is not commonly observed in wild type mice and has only been reported upon *H. felis* infection.[68]

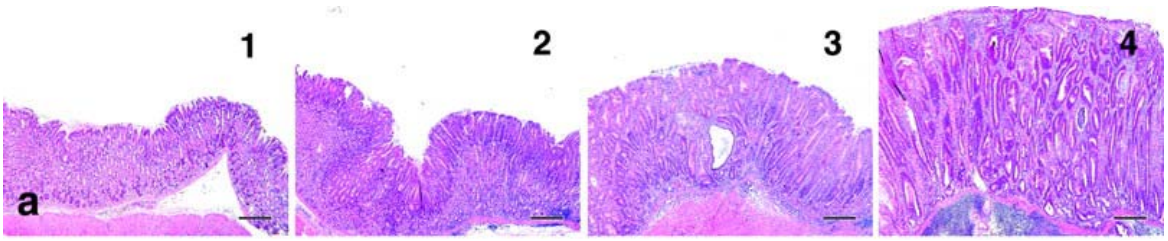


Fig. 4. Stepwise histopathology of *H. felis*-infected C57BL/6 mice. Normal tissue (1), acute inflammation (2), chronic inflammation with hyper- and dysplasia (3), and carcinoma (4). Hematoxylin and eosin; bar ≈ 300 µm. From [69].

In INS-GAS mice, genetically engineered to constitutively express gastrin under the insulin promoter, development of intraepithelial neoplasia is observed within months of *H. felis* or *H. pylori* infection.[8] BALB/c mice serve as model organism for MALT lymphoma because they develop gastric B-cell lymphomas closely resembling the human disease counterpart after long-term experimental infection with several *Helicobacter*.[70, 71]

The Mongolian gerbil model illustrates the whole pathologic process from gastritis to dysplasia and cancer upon *H. pylori* infection.[72] Other less currently used models include ferrets, guinea pigs, gnotobiotic piglets, rhesus monkeys[10] and beagle dogs.[73] Some of them are closer to the human situation in certain aspects. For example, in gerbils there is no strong selective pressure against the T4SS as in mice, and the guinea pig stomach shares several features with the human

stomach.[10] On the other hand, not many analysis tools are available and/or housing and handling is a lot more costly for these species. Nevertheless, they have also been studied besides mice as vaccine models.

1.4 Immune responses to *H. pylori*

1.4.1 Innate immunity

Besides adaptive immune mechanisms, infections are sensed by various so-called pattern recognition receptors (PRR) at the cell surface, endosomal membranes or in the cytosol. These detect certain conserved structures, so-called pathogen- or damage-associated molecular patterns (PAMPs/DAMPs), and activate measures to combat the infection and/or avert danger.

The role of one group of such receptors, membrane-bound Toll-like receptors (TLR), in detection of *H. pylori* by epithelial and innate immune cells has been addressed in several studies. These focused essentially on TLR2, TLR4 and TLR5, the receptors described to detect bacterial lipopeptides, lipopolysaccharide (LPS) from Gram-negative bacteria and flagellin, respectively.

The reaction of gastric epithelial cells to *Helicobacter* infection is in any case dependent on a functional T4SS and characterized by NF-κB activation with the subsequent production of proinflammatory chemokines such as GRO-α (or CXCL1), MCP-1 (CCL2), MIP-1α (CCL3) and IL-8, attracting neutrophils, monocytes and macrophages, and cytokines such as IL-1, IL-6 and TNF-α.[74-76] For this response, TLR2 seems to be required[77] whereas a requirement for TLR4[77-79] and TLR5[77, 80] is controversial.

Concerning innate immune cells, *H. pylori* has been reported to activate TLR2 and TLR4, leading to secretion of IL-1β and IL-6 or IL-12 and IL-10, respectively, in macrophages.[81] Others report that macrophages recognize *H. pylori* LPS via TLR4 but that TLR2 is the principal receptor recognizing whole bacterial cells and leading to release of cytokines such as IL-6 and MCP-1.[82]

Generally it seems that *H. pylori* LPS and flagellin only weakly interact with TLR4 and TLR5, respectively,[9, 80, 83] that TLR2 is weakly expressed in gastric epithelium, if at all,[9] and that therefore TLR2 on infiltrating cells represents the most important surface TLR for innate *H. pylori* detection.

TLR signaling, via the adaptor protein Myd88, has also been described to be necessary for activation of dendritic cells (DCs) by *H. pylori* and the initiation of a proinflammatory response.[84]

DCs recognize *H. pylori* lysate mainly via TLR2 and to a lesser extent TLR4. TLR2 signaling leaves an anti-inflammatory gene expression signature, however.[85] Importantly, TLR2 and TLR4 can be compensated in large parts by intracellular TLRs if intact bacteria are phagocytosed by the DCs. For example, endosomal TLR9 recognizes *H. pylori* DNA and initiates proinflammatory cytokines.[85] In addition, the intracellular receptor retinoic acid-inducible gene I (RIG-I), which gave name to the family of RIG-I like receptors (RLR), senses *H. pylori* RNA in a TLR-independent manner and induces type 1 interferons by activating interferon regulatory factors.[85]

NOD-like receptors (NLRs) represent a group of cytosolic PRRs, which have not been extensively studied in the context of *H. pylori* infection yet. So far, it has been demonstrated that *H. pylori* peptidoglycan injected into the host cell via the T4SS[86] and also outer membrane vesicles shedded from bacteria[87] are detected by NOD-1. NOD-1 subsequently signals via type I interferons and the ISGF3 pathway[88] and/or induces antimicrobial peptides.[89]

Other NLRs, the best characterized being NALP3 and NLRC4 (or IPAF), are integral components of inflammasomes. These intracellular signaling complexes recognize various PAMPs and DAMPs, which leads to activation of the enzyme caspase-1 and to the processing and release of the cytokines IL-1β and IL-18.[90] IL-1β has proinflammatory activity and a known prominent role in *H. pylori* infection and gastric cancer. IL-18 has not been attributed a clear function in this context so far. Many Gram-negative and Gram-positive bacteria activate inflammasomes.[91-94] While the NALP3 inflammasome recognizes a wide range of signals, NLRC4 mainly detects flagellin of Gram-negative bacteria injected into the cytosol by type III or IV secretion systems.[95, 96] Astonishingly, the possible activation of the inflammasome by *H. pylori* has not been addressed in detail yet. Activation of caspase-1 by *H. pylori* LPS in macropohages has been shown[97] but further studies are needed to extend this finding to whole bacteria and other cell types, to identify the NLR(s) possibly detecting *H. pylori* components and to define the relevance of inflammasome activation in *in vivo* models.

Taken together, innate mechanisms recognizing *H. pylori* are being incrementally elucidated but their respective relevance for the overall response still need to be better defined.

1.4.2 Adaptive immunity

The adaptive immune responses towards *H. pylori* are subject to ongoing investigations. Antibody responses were initially believed to be important players as they are in other mucosal infections. Indeed, *H. pylori* elicits prominent mucosal and serum immunoglobulin responses

and *H. pylori*-specific serum antibody titers serve as markers of infection in large epidemiological studies. In the meantime, however, T cell mediated immunity is regarded as the vital adaptive immune mechanism towards *H. pylori* infection (see also section 1.5.2).

H. pylori infection leads to recruitment of $CD4^+$ and $CD8^+$ T cells to the gastric mucosa in mice[98] and humans.[99] Even though shown to produce IFN-γ in response to *H. pylori* antigens,[100] no prominent role for $CD8^+$ T cells has been described, except in the absence of $CD4^+$ T cells.[98] The $CD4^+$ T cell response induced by the infection is clearly polarized to the Th1 type. The Th1-promoting cytokines IL-12 and IFN-γ are up-regulated in the gastric mucosa of infected patients[101, 102] and mice[103, 104] and preferentially induced by *H. pylori* components in DCs and peripheral blood mononuclear cells.[105, 106] Th1 cells and cytokines have been reported to drive *H. pylori*-induced gastritis and pathology.[104, 107, 108] Th2 responses, characterized by the cytokines IL-4 and IL-5, in contrast, have been described to act against *H. pylori* induced gastritis.[107] A role in attenuation of bacterial loads has been discussed for both subsets (see section 1.5.2). An involvement of the IL-17-producing T helper subset (Th17) in the context of *H. pylori* infection seems likely since Th17 cells have been implicated in protective immunity against other pathogens[109, 110] and in chronic inflammatory conditions.[111-113] Therefore detailed studies with regard to this cell type in *H. pylori* models are warranted.

Even though rather strong T cell responses are induced, these do not manage to clear *H. pylori* from the gastric mucosa. *H. pylori* has evolved strategies to evade and downregulate the immune responses of its host. One such mechanism is the induction of regulatory T cells (Tregs). Tregs infiltrate the gastric mucosa of *H. pylori* infected individuals compared to uninfected individuals.[114, 115] Depletion of Tregs in *H. pylori* infected mice leads to enhanced inflammation, gastric pathology and reduced bacterial colonization.[115, 116] How *H. pylori* induces such Treg responses that dampen local immunity and enable the bacteria to survive is not entirely clear. Surprisingly, B cell deficient mice have been show to have reduced colonization along with increased gastric $CD4^+$ T cell levels and inflammation after several months of infection with *H. pylori*, suggesting a regulatory role of this cell type.[117] On the other hand, B cells are at the origin of *H. pylori*-driven MALT lymphoma, which could in part be due to suppression of B cell apoptosis by low-level *H. pylori* infection.[118] Moreover B cells could be implicated in the development of pathogenic autoimmunity by producing autoreactive antibodies.[119] Therefore B cells cannot be attributed an overall detrimental or beneficial role in *H. pylori* infection.

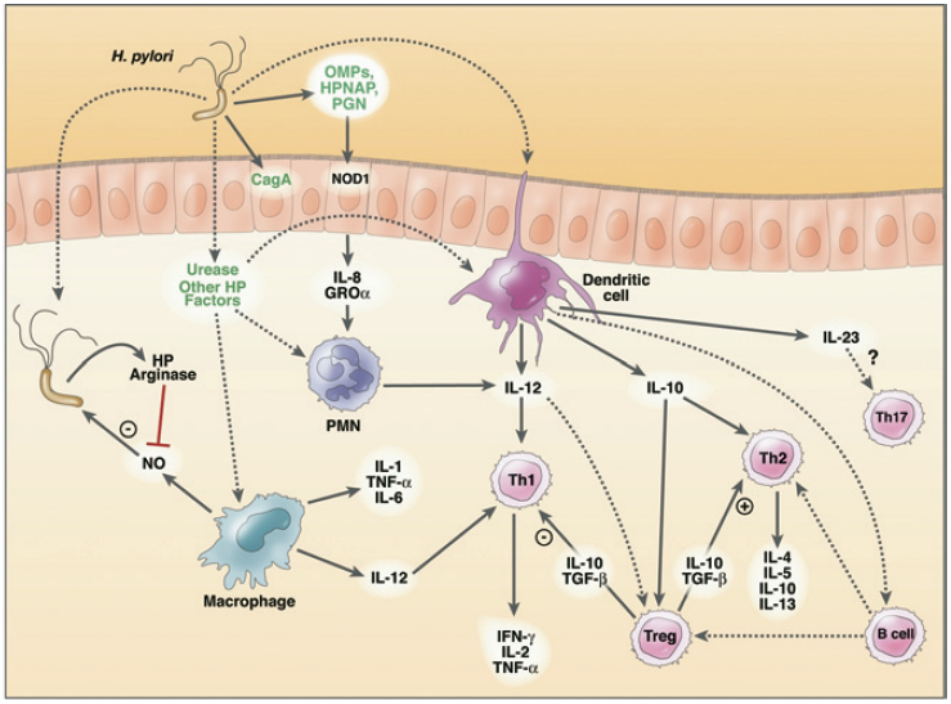


Fig. 5. Immune responses to *H. pylori*. From [120].

1.5 Towards a *H. pylori* vaccine

1.5.1 Vaccination approaches in mouse and man

Almost immediately after the discovery of *H. pylori*, in parallel to developing eradication therapies, researchers went on a quest for possible vaccination strategies. Regimens have been tested that would prevent initial colonization by *H. pylori* or work against the infection in a therapeutic manner.

Routes

Many efforts concentrate on oral vaccination regimens since this route is believed to possibly be crucial in order to induce efficient mucosal immunity.[17, 121-129] Also intranasal delivery as another way of mucosal vaccination has been attempted.[123, 130] Systemic administration is nevertheless the ultimative goal for a human vaccine because it promises the best compliance and safety, and therefore its feasibility is being investigated with several approaches as well.[131-135]

Antigens

Most studies utilize bacterial whole cell sonicate or lysate as antigen. Recombinant *H. pylori* urease or its two structural subunits UreA and UreB, respectively, were used formulated with an adjuvant[122, 123, 129, 131] or in form of an attenuated recombinant *Salmonella* vaccine strain expressing the antigen.[136-138] Other antigens tested successfully so far include CagA, VacA,[122, 125] NAP,[17, 133] HpaA,[139-141] catalase[128] and the heat shock proteins HspA and HspB.[121, 140]

Adjuvants

The gold standard adjuvant used for *H. pylori* vaccination studies in animal models is Cholera toxin (CT), the exotoxin of *Vibrio cholerae*, which has very potent mucosal adjuvant activity and is usually administered orally. It is toxic for humans, however, since its uptake into intestinal epithelial cells leads to severe diarrhea. The same is the case with *Escherichia coli* heat-labile toxin (LT), which has even shown activity in a parenteral *Helicobacter* vaccine.[142] Therefore, several mutant, non-toxic derivatives of CT and LT have been generated and tested for adjuvanticity in oral regimens.[122, 125] One study used a recombinant fusion peptide consisting of a UreB epitope and the cholera toxin B subunit, which was liposome-encapsulated and administered orally.[143]

CpG oligodeoxynucleotides (CpG-ODN), potent inducers of Th1 responses, which might have potential for approval as human adjuvant,[144] have been given orally,[145] intranasally[130] or subcutaneously[135] alone[146] or together with whole cell sonicate.[130, 135] Complete Freund's Adjuvant (CFA), consisting of inactivated and dried mycobacteria, administered systemically with lysate was partially protective in neonatal and adult mice,[132, 134] is however not an option for human use due to its strong toxicity. Aluminium hydroxide (AlOH) is the adjuvant commonly used for human vaccines. Preventive vaccination using AlOH in mice[132] and also therapeutic vaccination in Beagle dogs[133] has been reported to be able to limit and in some cases even eradicate infection. However, in another study the protective effect of intramuscular AlOH vaccination has been described to be inferior to that of an oral vaccine containing mutant LT.[140]

Alternative approaches

Some further strategies have been tested to circumvent the necessity of employing an adjuvant or needle use with vaccination. For instance, bone marrow derived DCs were stimulated *ex vivo* with *H. pylori* and administered intraperitoneally with or without concomitant Treg depletion.[147]

A lipid-based matrix containing *H. pylori* sonicate was utilized for transcutaneous immunization.[148] Though being innovative, such approaches have brought only limited success so far.

Generally it must be noted that most of the described vaccines are successful in reducing *H. pylori* loads to a significant extent compared to infected controls. However, sterilizing immunity is only rarely achieved. In some cases where this has been reported the employed colonization read-out, such as rapid urease test or histological staining, actually might have been not accurate enough to detect residual colonization, or challenge infection might have been not very effective since also some of the infected controls were able to clear.

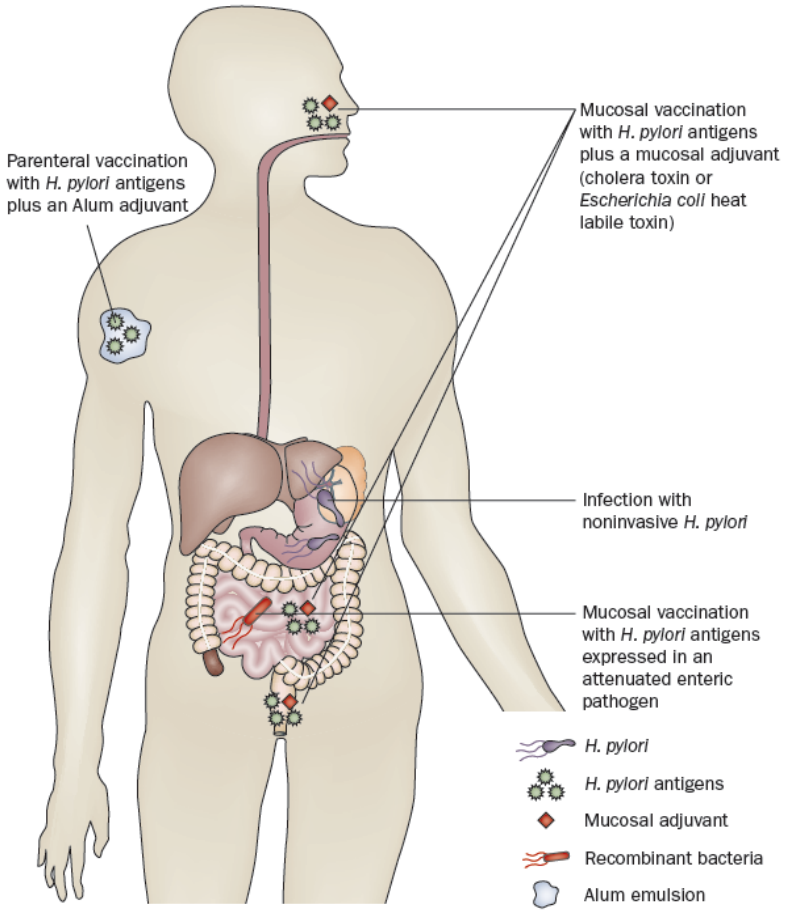


Fig. 6. Overview of the most commonly tested *H. pylori* vaccination strategies and routes in human and animal models. From [149].

Human studies

To date, only a few studies in human volunteers have been conducted, with modest outcome. Safety and immunogenicity trials were performed with recombinant *Salmonella typhi*[138] or

Salmonella enterica[136, 137] expressing UreA and UreB, a whole cell vaccine with mutant LT adjuvant[150] and UreB adjuvanted with LT via the oral[151, 152] or rectal route.[153] Drawbacks of these studies were side effects like diarrhea,[151] fever and vomiting,[150] and/or insufficient immunogenicity. In a challenge trial after vaccination with *Salmonella enterica* expressing *H. pylori* urease or the protein HP0231 the achieved protection was not satisfactory.[154] The most recent approach using three recombinant antigens, CagA, VacA and NAP, administered intramuscularly with AlOH showed good safety and immunogenicity.[155] It will be interesting to see the results of ongoing challenge trials.

1.5.2 Mechanisms of vaccine-induced protection

The protective mechanisms after vaccination have been subject of much debate and are still not entirely clarified. Since antibodies are generally believed to be required to combat infections by extracellular bacteria, several groups hypothesized initially that antibodies, be it secretory IgA or local IgG responses,[156] were needed for protection against *H. pylori*. Indeed, passive oral immunization with specific antibodies has been shown to prevent or suppress *H. felis* infection in mice and gerbils.[157-159] Moreover, suckling mice are protected by antibodies in the milk of a previously immunized dam,[160] and *H. pylori*-specific IgA in the milk of breast-feeding mothers delays colonization of their infants.[161] However, it has become clear that cell-mediated immunity is essential for the active reduction of bacterial loads, and there is no requirement for antibodies.[117, 123, 132, 162, 163] In contrast, as mentioned above, antibodies and B cells themselves have even been suggested to facilitate the bacterial colonization.[117, 164] The absence of vaccine-induced protection in mice lacking major histocompatibility complex class II (MHCII) clearly pointed out the requirement of $CD4^+$ T cells.[123, 165] Despite suboptimal results, the first human challenge trial confirmed this notion in that strong T cell responses elicited in the volunteers correlated with reduction of *H. pyl*ori counts.[154]

There is disagreement in the literature concerning the type of T helper cell response required. The most widely used adjuvant for *H. pylori* vaccination in animal studies, CT, has actually been described to induce mainly Th2 responses and to inhibit Th1 responses.[166-170] In contrast, Th2 responses seem dispensable for protection after oral vaccination using CT in conjunction with *H. pylori* antigens since C57BL/6 mice lacking IL-4 or IL-5 are protected to a normal extent.[162] In fact, a requirement for the Th1-promoting cytokine IL-12 has been described since knockout mice displayed a lack of protection.[171, 172] Whereas Akhiani et al. claim that efficient vaccination

further needs IFN-γ and is mediated by Th1 cells,[171] Garhart et al. do not see a requirement for IFN-γ.[172] Taylor et al. report as well that protection after intramuscular CT vaccination correlates with *H. pylori* specific IL-12 levels, but neither with IFN-γ nor IL-4.[173] Various other adjuvants – with established Th1 or Th2 polarizing activity or undefined mode of action – have been analyzed. For instance, colony forming units (CFU)-reducing immunity after vaccination with CpG-ODN, known for their ability to induce nearly entirely Th1-biased immune responses, was associated with strong IFN-γ and IgG2a levels and abolished in IFN-$\gamma^{-/-}$mice.[130] Taylor et al. claim that a Th2 polarizing vaccine, consisting of CT and LPS-depleted *H. pylori* sonicate, induced more local and systemic responses than a Th1 polarizing vaccine, containing CpG and normal sonicate, but that only the latter one was protective.[174]

On the contrary, Guy et al. found that amongst several adjuvants tested in parallel the one inducing strong mixed Th1/Th2 responses was most efficient.[131] Moreover, it has been claimed that IL-5-producing T cells elicited by vaccination with AlOH confer partial protection comparably to IFN-γ-producing T cells generated with a CFA vaccine in mice[132] and that protective immunity in gerbils vaccinated using either AlOH or LT is associated with a predominant Th2 response.[140] One group reported that only a Th2, but not a Th1, cell line specific for *H. pylori* could reduce bacterial loads upon adoptive transfer into subsequently infected naive recipients, and that infected interleukin 4-deficient mice, on a mixed 129Sv x C57BL/6J background in this case, exhibited increased numbers of bacteria.[107] Another group found differences in the inherent clearance capability of mice lacking IL-4 depending on the strain: while IL-$4^{-/-}$ mice had reduced colonization in the C57BL/6 background, slight over-colonization was observed in the Balb/c background.[175] Even though this suggested a role for Th2 cells in reducing bacterial loads at least in Balb/c mice, depletion of Tregs in (not previously vaccinated) infected Balb/c mice led to an enhanced Th2 response but no change in bacterial levels.[176]

Whilst the effector T cells involved in vaccine-induced protection have attracted a lot of attention, the final effector mechanisms elicited by these T cells that lead to at least partial eradication of *H. pylori* from the stomach are unclear and subject of ongoing research. Besides phagocytes recruited to the gastric mucosa by chemokines and activated by cytokines, epithelial defense mechanisms come into consideration such as secretion of complement or antimicrobial substances.

2. AIMS OF MY STUDIES

2.1 Elucidating the mechanism of action of a successful *H. pylori* vaccine

Given the rather unclear data on requirements and correlates of vaccine-induced protective immunity against *H. pylori*, we set out to systematically address the role of different innate and adaptive immune system components as well as the gastric epithelium in an efficient vaccine regimen. Therefore we used various knock-out strains or depleted certain cell types in the gold standard murine vaccination model using Cholera toxin plus whole cell sonicate of *H. pylori* SS1.

2.2 Identifying alternative approaches for a human *H. pylori* vaccine

In parallel, we went on a quest for an alternative adjuvant at least as efficient as Cholera toxin that would be suitable for human use and ideally act by activating exactly the mechanisms found to be important for protection. We tested several adjuvant candidates and immune activators alone or in combination, different mucosal and systemic administration routes as well as a recombinant antigen in terms of their potential to significantly reduce *H. pylori* loads.

2.3 Addressing the concern of persistent gastritis after vaccination

Since the available literature is incomprehensive concerning the possibility of a suboptimal vaccination strategy to actually aggravate immunopathology caused by infection, we examined this issue by analyzing immunized mice at different time points after challenge infection in terms of bacterial colonization as well as epithelial pathology compared to naive infected animals.

2.4 Investigating the role of caspase-1 in the context of *H. pylori* pathogenesis

Having observed that mice lacking caspase-1 are less well protected in the vaccine model but display an increased spontaneous clearance potential we endeavored to comprehensively study the function of this central player of the inflammasome and its downstream mediators, the cytokines IL-1β and IL-18, with regard to *Helicobacter* infection control and pathogenesis.

3. RESULTS

The results obtained during my thesis are represented by the following published articles and a manuscript currently in preparation. They are discussed in conjunction with some unpublished observations in the subsequent section.

In the first publication we highlight the importance of IFN-γ-producing $CD4^+$ T cells elicited by *Helicobacter* infection for reduction of bacterial loads but at the same time for the formation of premalignant gastric lesions. We show that inflammation and immunopathology are aggravated in immunized animals unable to completely clear the infection compared to infected naive mice. The second article recapitulates the results of the first publication and additionally shows that post-immunization pathology is further increased at a later time point after challenge infection.

The third manuscript points out the mycobacterial adjuvant CAF01 as potent candidate for human *H. pylori* vaccination, eliciting strong Th1/Th17 responses via systemic delivery routes. The superiority of local memory $CD4^+$ T cell, neutrophil and mast cell infiltration over systemic antibody titers as immunological correlates of protection is highlighted. Besides, we identify dendritic cells rendered tolerogenic by *H. pylori* as one main obstacle to a truly efficient vaccine.

The fourth article analyzes the *H. pylori* CagA protein in terms of immunogenicity and suitability of use as vaccine antigen. We show that oral or systemic CagA vaccination is unable to reduce bacterial loads after challenge with the $CagA^+$ *H. pylori* strain PMSS1, despite inducing a strong immune response and considerable pathology, and therefore is not a valid strategy.

In the last manuscript we emphasize the previously unappreciated impact of the inflammasome and its main effector, the IL-1β- and IL-18-converting enzyme caspase-1, on the outcome of *Helicobacter* infection. We show how caspase-1, depending on the setting and via its substrates, is balancing tolerance and immunity towards *Helicobacter* and thereby controls pathology.

For a further publication to which I have contributed but which I do not discuss in the scope of this dissertation, please refer to the appendix. It deals with the pharmacological inhibition of pathologic Th1 responses towards *Helicobacter* by interfering with the COX-2/PGE2 pathway.

3.1 The CD4$^+$ T cell-mediated IFN-γ response to *Helicobacter* infection is essential for clearance and determines gastric cancer risk

article published in Journal of Immunology, 2009

authors: Ayca Sayi*, Esther Kohler*, Iris Hitzler*, Isabelle Arnold, Reto Schwendener, Hubert Rehrauer and Anne Müller (* equal contribution)

contributions: I contributed the figures 4 and 6, generated all cytokine real-time PCR data and helped to edit and to revise the figures and the manuscript. AM, AS and EK contributed the other data, AM wrote the manuscript. IA provided a cell line, RS performed injections, HR assisted with microarray analysis and statistics.

The CD4$^+$ T Cell-Mediated IFN-γ Response to *Helicobacter* Infection Is Essential for Clearance and Determines Gastric Cancer Risk[1]

Ayca Sayi,[2]* Esther Kohler,[2]* Iris Hitzler,[2]* Isabelle Arnold,* Reto Schwendener,* Hubert Rehrauer,[†] and Anne Müller[3]*

Chronic infection with the bacterial pathogen *Helicobacter pylori* is a risk factor for the development of gastric cancer, yet remains asymptomatic in the majority of individuals. We report here that the C57BL/6 mouse model of experimental infection with the closely related *Helicobacter felis* recapitulates this wide range in host susceptibility. Although the majority of infected animals develop premalignant lesions such as gastric atrophy, compensatory epithelial hyperplasia, and intestinal metaplasia, a subset of mice is completely protected from preneoplasia. Protection is associated with a failure to mount an IFN-γ response to the infection and with a concomitant high *Helicobacter* burden. Using a vaccine model as well as primary infection and adoptive transfer models, we demonstrate that IFN-γ, secreted predominantly by CD4$^+$CD25$^-$ effector T$_H$ cells, is essential for *Helicobacter* clearance, but at the same time mediates the formation of preneoplastic lesions. We further provide evidence that IFN-γ triggers a common transcriptional program in murine gastric epithelial cells in vitro and in vivo and induces their preferential transformation to the hyperplastic phenotype. In summary, our data suggest a dual role for IFN-γ in *Helicobacter* pathogenesis that could be the basis for the differential susceptibility to *H. pylori*-induced gastric pathology in the human population. ***The Journal of Immunology,*** **2009, 182: 7085–7101.**

The Gram-negative bacterium *Helicobacter pylori* colonizes the human stomach and is associated with a range of gastric and duodenal disorders including chronic gastritis, stomach and duodenal ulcers, MALT lymphoma, and gastric adenocarcinoma (1–5). Whereas ~20% of infected individuals develop symptomatic disease, the large majority remains asymptomatic despite persistent, often lifelong colonization with the bacterium. This wide diversity in colonization outcome has been attributed to strain differences (Western vs Eastern *Helicobacter* strains), genetically determined host susceptibility, and lifestyle factors such as nutrition and hygiene (6). However, no single universal explanation has yet been found to hold true across all populations studied, and it is likely that a combination of multiple factors determines infection outcome.

Roughly 1–2% of infected individuals develop gastric adenocarcinoma, the fourth most common diagnosed cancer and second most common cause of cancer-related deaths worldwide (7, 8). Gastric cancer is traditionally classified into two subtypes based on its histological architecture (9). The intestinal type, characterized by cohesive growth of cells in quasi-glandular structures, has been presumed to arise secondarily from intestinal metaplasia, whereas the diffuse type, in which tumor cells are dispersed and generally not organized into glandular structures, is thought to arise directly from nonmetaplastic foveolar or mucous neck cells (9, 10). Little is known about the molecular pathogenesis of either type of gastric cancer. This may at least partly be due to its diversity; in fact, in a transcriptional profiling study comparing the transcriptomes of >90 gastric cancer samples, the most striking feature was their inherent molecular heterogeneity (11). Both subtypes of gastric cancer have been linked to *Helicobacter pylori* in epidemiological studies. The intestinal type in particular progresses via a sequence of premalignant lesions, chronic gastritis, atrophic gastritis, intestinal metaplasia, and dysplasia- (12–14), of which each has been independently linked epidemiologically to infection with the pathogen. The chronic inflammation induced by *H. pylori* very likely provides the setting in which gastric cancer initiation can occur.

Mouse models of *H. pylori* infection have been widely used to study certain aspects of its pathogenesis, such as the virulence factors allowing it to colonize the hostile niche of the gastric environment (15). For investigating the mechanisms underlying *Helicobacter*-induced gastric pathology, however, the closely related *Helicobacter felis* provides a more useful model system. In T$_H$1-polarized mice such as the C57BL/6 strain, *H. felis* induces a rapid proinflammatory and adaptive immune response that is reminiscent of the human setting (16, 17). In the T$_H$2-polarized BALB/c strain of mice, in contrast, the infection induces gastric-organized lymphoid tissue (MALT), which can progress to malignant gastric B cell lymphoma (18–21). The most severe outcome reported for experimental murine *H. felis* infection is in situ carcinoma, which develops if infected mice are maintained on a high salt diet or are genetically modified to be hypergastrinemic due to expression of the human gastrin transgene under the control of the insulin promoter (22, 23).

*Institute of Molecular Cancer Research and [†]Functional Genomics Center Zurich, University of Zurich, Zurich, Switzerland

Received for publication October 1, 2008. Accepted for publication March 23, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This study was funded by grants from Schweizer Nationalfonds (3100A0-113452), the UBS Foundation (BA29 S8Q7-DZZ 969/A), and the Nils and Desiree Yde Stiftung to A.M. Additional funding was supplied by the University Research Priority Program in Systems Biology/Functional Genomics and the Institute of Molecular Cancer Research.

[2] A.S., E.K., and I.H. contributed equally to this work.

[3] Address correspondence and reprint requests to Prof. Dr. Anne Müller, Institute of Molecular Cancer Research, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland. E-mail address: mueller@imcr.unizh.ch

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0803293

In humans, the *H. pylori*-infected gastric mucosa is characterized by high levels of proinflammatory cytokines including IL-8, IL-1β, TNF-α, and IL-6 (24, 25). The signature cytokines of T_H1-polarized cellular immune responses, IFN-γ, IL-12, and IL-18, are also present at elevated levels (26, 27). Despite the production of large amounts of both types of cytokines and the efficient recruitment of neutrophil granulocytes, macrophages, and various lymphocyte populations to the site of infection, the immune response of the host is typically inadequate to clear the infection. Rather, above normal production of inflammatory cytokines, for example due to polymorphisms in their promoter regions, has been linked to an increased risk of gastric cancer (25, 28, 29).

In this study, we demonstrate that IFN-γ plays a key (dual) role in the response to *Helicobacter* infection. Using experimental infection models in wild-type and various knockout strains as well as adoptive transfer and vaccination models, we show that IFN-γ produced by $CD4^+CD25^-$ effector cells is crucial for the control of *Helicobacter* infection on the one hand, and induces preneoplastic changes of the gastric mucosa on the other. We further show that even genetically virtually identical animals differ in their ability to mount strong IFN-γ responses to the infection and that the extent of the IFN-γ response determines gastric cancer risk.

Materials and Methods

Mice and bacterial infections

All mice (C57BL/6, IFN-$\gamma^{-/-}$ Bl6, TCR-$\beta^{-/-}$ Bl6, and Rag-$1^{-/-}$ Bl6) were purchased from Charles River Laboratories and bred at a University of Zurich specific pathogen-free facility. Mice were housed in individually ventilated cages and infected with *Helicobacter* at 5–6 wk of age. All animal experiments were performed in accordance with institutional policies and have been reviewed and approved by the cantonal veterinary office.

The *Helicobacter* strains used in this study were *H. pylori* Sydney strain 1 (SS1, originally obtained from A. Lee, University of New South Wales, Sydney, Australia (30)) and *H. felis* CS1 (ATCC 49179). *H. pylori* was grown on solid medium on horse blood agar containing 4% Columbia agar base (Oxoid; Basingstoke), 5% defibrinated horse blood (HemoStatLabs), 0.2% β-cyclodextrin, 5 μg/ml trimethoprim, 8 μg/ml amphotericin B, 10 μg/ml vancomycin, 5 μg/ml cefsulodin, and 2.5 U/ml polymyxin B sulfate (all from Sigma-Aldrich) at 37°C for 2 days under microaerophilic conditions. For liquid culture, *H. pylori* was grown in *Brucella* broth (Difco) containing 10% FBS (Life Technologies) with shaking in a microaerobic atmosphere at 37°C. Before mouse infections, bacteria were inoculated from frozen stocks onto horse blood plates, incubated for 2 days, and then expanded onto fresh plates and grown for 24 h. Bacteria harvested from these plates were used to inoculate overnight liquid cultures. Cultures were routinely assessed by light microscopy for contamination, morphology, and motility. Infections were performed by oral gavage with 10^8 bacteria in 100 μl of suspension in *Brucella* broth. *H. felis* was grown similarly, except that all antibiotics except for trimethoprim and amphotericin were omitted from the blood plates.

Generation and culture of an immortalized murine primary gastric epithelial cell line

Primary cultures of murine gastric epithelial cells were prepared according to a protocol developed by Fujikawa et al. (31). Two days after seeding, the cells were immortalized based on a procedure first described by Tucker et al. (32). Cell clusters were infected with a Moloney murine leukemia virus carrying the temperature-sensitive SV40 large T Ag in the presence of 8 μg/ml polybrene. G418-resistant clones were picked 3 wk later. The epithelial origin of the clone of interest was confirmed by E-cadherin staining. For assessment of epithelial IFN-γ responses, the cells were treated with 0.1, 1, and 10 μg/ml recombinant IFN-γ for 24 h. RNA was extracted and purified over RNeasy columns and subjected to transcriptional profiling using Affymetrix Genechips.

Immunizations

Wild-type and IFN-$\gamma^{-/-}$ mice were vaccinated four times at weekly intervals by oral gavage of 1 mg of whole cell sonicate of *H. pylori* strain SS1 along with 10 μg of cholera toxin as adjuvant (List Biologicals). Two weeks after the last immunization, immunized and nonimmunized control mice of either genotype were infected with 10^8 CFU of *H. pylori* SS1. Mice were sacrificed 2 or 7 wk after challenge and bacterial colonization was assessed by colony count assay as well as *ureB*-specific PCR on genomic DNA extracted from stomach samples.

Purification and adoptive transfer of $CD4^+CD25^-$ T cell populations

$CD4^+CD25^-$ T cells were purified from single- cell suspensions of freshly isolated donor spleens. Immunomagnetic sorting was performed using a $CD4^+CD25^+$ T cell purification kit from R&D Systems according to the manufacturer's instructions. Cell preparations were washed, resuspended in cold RPMI 1640 medium and adoptively transferred into immunodeficient (Rag-$1^{-/-}$ or TCR-$\beta^{-/-}$ Bl6) hosts by i.v. injection in a 200-μl volume. If not noted otherwise, 300.000 cells were transferred. The recipients were routinely infected 1 day after adoptive transfer. Uninfected as well as infected mice that had not received cells served as controls. Where indicated, $CD4^+CD25^-$ T cell recipients were not infected and served as additional controls. For depletion of neutrophils, mice were treated with 250 μg of anti-GR-1 Ab (clone RB6-8C5, provided by J. Huelsken, Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland) three times weekly for the duration of the experiment.

Preparation of gastric tissues and assessment of colonization and pathology

Mice were killed at 2 wk, 1 mo, or 3 mo after infection by CO_2 inhalation; the stomachs were retrieved and cleaned and the forestomach was removed. The remaining piece containing the corpus and antrum was opened along the lesser curvature and spread out in the form of a trapeze. The tissue was then dissected longitudinally (i.e., from the forestomach/corpus junction down to the antrum/duodenum junction) into six equal, parallel pieces that comprise close to identical proportions of antral and corpus tissue. Of every stomach, the same section (i.e., the right-most, left-most etc.) was designated for the same downstream processing (DNA extraction, histology, etc.) to minimize sampling error. For quantitative assessment of *H. pylori* colonization, one section of each stomach was transferred to a tube containing *Brucella* broth and homogenized with an Ultra Turrax homogenizer (John Morris Scientific). Serial dilutions were plated on horse blood plates to determine bacterial loads. For quantitative PCR-based assessment of either *H. felis* or *H. pylori* colonization, another sixth of each stomach was subjected to genomic DNA isolation using a DNeasy blood and tissue kit (Qiagen). Genomic whole stomach DNA served as a template for PCR using primers directed against urease and flagellin genes. Primer sequences and PCR conditions are listed in Table I. For quantitative assessment of colonization according to a protocol first described by Stoicov et al. (33), 80 ng of extracted DNA was used for real-time PCR (LightCycler; Roche) with the LightCycler 480 SYBR Green I master kit (Roche). Standards were made by sequential 10-fold dilutions of purified *H. felis* DNA producing a range from 500,000 to 5 copies per reaction. This is based on the premise that 2 fg of *H. felis* chromosomal DNA is equivalent to one copy of the *H. felis* genome (33). Each sample was analyzed in triplicate. For histology, two additional stomach sections of one-sixth each were either fixed in 10% neutral-buffered formalin before paraffin embedding or were embedded in cold OCT medium for cryosectioning. Consecutive paraffin sections were stained with H&E, Giemsa, periodic acid-Schiff, and Alcian blue for grading of histopathological changes. One to two longitudinal sections per mouse spanning the length of the stomach from the forestomach/corpus junction to the antrum/duodenum junction were scored with regard to four histopathological parameters (chronic inflammation, gastric atrophy, intestinal metaplasia, and mucus pit cell/epithelial hyperplasia) based on the features described in the updated Sydney classification (34). We attributed scores on a scale of 0–6 as proposed by Chen et al. (35). Specifically, the definition of scores was as follows for the four parameters evaluated. Scores for chronic inflammation were: 0, none; 1, some infiltrates; 2, mild (few aggregates in submucosa and mucosa); 3, moderate (several aggregates in submucosa and mucosa); 4, marked (many big aggregates in submucosa and mucosa); 5, nearly the entire mucosa contains a dense infiltrate; and 6, entire mucosa contains a dense infiltrate. Scores for atrophy were: 0, none; 1, foci where a few gastric glands are lost or replaced; 2, small areas in which gastric glands have disappeared or been replaced; 3, <25% of gastric glands lost or replaced; 4, 25–50% of gastric glands lost or replaced; 5, >50% of gastric glands lost or replaced; and 6, only a few small areas of gastric differentiated glands remaining. For intestinal metaplasia, the scores were: 0, none; 1, only one crypt replaced by intestinal epithelium; 2, one focal area (one to four crypts) replaced; 3, two separate foci with metaplasia; 4, multiple foci; 5, >50% of gastric epithelium replaced by i.e.; and 6, only a few small areas of gastric epithelium are not replaced by intestinal epithelium. For hyperplasia, the scores were: 0,

Table I. *Primers and PCR conditions used in this study*

Gene/Transcript	Nucleotide Sequence (5′–3′)	T_m (°C)	PCR Cycles
flaB (*H. felis*)	Fw[a]: TTCGATTGGTCCTACAGGCTCAGA	55	34
	Rv: TTCTTGTTGATGACATTGACCAACGCA		
ureB (*H. felis*)	Fw: ATGAAACTAACGCCTAAAGAACTAG	57	33
	Rv: GGAGGATAAAGTGAATATGCGT		
ureB (*SS1*)	Fw: CGTCCGGCAATAGCTGCCATAGT	58	30
	Rv: GTAGGTCCTGCTACTGAAGCCTTA		
IFN-γ (convent.)	Fw: GGTGACATGAAAATCCTGCAGAGC	58	35
IFN-γ (real time)	Rv: TCAGCAGCGACTCCTTTTCCGCTT	55	50
	Fw: CATGGCTGTTTCTGGCTGTTACTG		
	Rv: GTTGCTGATGGCCTGATTGTCTTT		
IP-10	Fw: CCTATCCTGCCCACGTGTTGAG	55	33
	Rv: CGCACCTCCACATAGCTTACA		
MIP-2	Fw: AGTTTGCCTTGACCCTGAAGCC	55	35
	Rv: GGAACTAGCTACATCCCACCCA		
GAPDH	Fw: GACATTGTTGCCATCAACGACC	55	32
	Rv: CCCGTTGATGACCAGCTTCC		

[a] Fw, Forward; Rv, reverse.

none; 1, single glands (next to infiltrate); 2, one focal area/one to four crypts (mild); 3; one to three foci; 4, multiple foci; 5, >50% of glands affected; and 6, only few small nonhyperplasic areas.

Immunohistochemistry

For staining of cells in the S phase (actively proliferating cells) by BrdU incorporation, animals received 10 μl of 10 mM BrdU/g of bodyweight i.p. 60 min before sacrifice. Paraffin sections of 4 μm were stained with anti-BrdU Ab according to instructions provided with an In Situ Cell Proliferation Kit (Roche) and counterstained with a proliferating cell nuclear Ag-specific biotinylated Ab (PCNA;[4] 1/10 dilution, mouse clone PC10; Zymed Laboratories). Detection was either performed histochemically with HRP-coupled streptavidin (Jackson ImmunoResearch Laboratories) and diaminobenzidine substrate (Research Genetics/Invitrogen) or with Alexa Fluor 594-coupled streptavidin for fluorescence microscopy. For staining of the CD4 surface marker, frozen 5-μm sections were dried overnight, fixed briefly in acetone, dried for several more hours, and stained with a FITC-coupled CD4-specific Ab (1/25, rat clone RM4-5; BD Pharmingen/BD Biosciences). Parietal cells were visualized by Alexa Fluor 594-coupled streptavidin, which binds to the abundant biotin found in this cell type. Cryosections were mounted in 4′,6-diamidino-2-phenylindole-containing mounting medium to visualize nuclei in blue (Vector Laboratories).

Semiquantitative and quantitative assessment of gastric cytokine responses

For conventional and real-time RT-PCR of IFN-γ, IFN-inducible protein 10 (IP-10), MIP-2, and GAPDH, total RNA was isolated from one-sixth of every stomach (antrum and corpus) using RNeasy Mini columns (Qiagen). In brief, 1.5 μg of total RNA was used for first-strand cDNA synthesis with Superscript Reverse Transcriptase III (Invitrogen). The resulting cDNA served as a template for PCR (for conditions and primer sequences, see Table I). IFN-γ-specific real-time PCR (LightCycler; Roche) was performed with a LightCycler 480 SYBR Green I master kit (Roche). Absolute values of IFN-γ expression were normalized to GAPDH expression. For quantitative assessment of IFN-γ gastric protein levels, an IFN-γ ELISA (Quantikine Immunoassay System; R&D Systems) was performed using gastric mucosal extracts according to the manufacturer's instructions.

Gene expression profiling using Affymetrix Genechips and data analysis

For transcriptional profiling analysis, total RNA was isolated from homogenized samples of scraped mucosa from one-sixth of every stomach (antrum and corpus) using TRIzol reagent (Invitrogen). After the extraction procedure, the RNA was cleaned up by a RNeasy Mini Kit (Qiagen) and RNA integrity was verified by capillary gel electrophoresis using a Bioanalyzer (Agilent Technologies). Reverse transcription was performed with 2 μg of total RNA and the obtained cDNA was subjected to synthesis and biotin labeling of cRNA using Affymetrix GeneChip One-Cycle Target Labeling and Control Reagents according to the manufacturer's protocol. Ten micrograms of the cRNA/sample was hybridized for 16 h at 45°C to Affymetrix GeneChip Mouse Genome arrays 430.2, which contain ~45,000 probes representing the entire mouse genome. After hybridization, the arrays were washed and stained with streptavidin-conjugated PE in a GeneChip Fluidics Station 400 (Affymetrix) and were scanned with a Hewlett-Packard Scanner. Raw gene expression data generated by the GeneChip Operating Software (Affymetrix) were normalized for all probe sets on the array using the Robust Multichip Average method. The expression values were imported into the Resolver application (Rosetta Biosoftware) for hierarchical clustering. Sample clustering was performed based on the present genes only, with a gene being called present if its average expression in the study was above 25. We used the correlation of the log 2 expression values as the measure of similarity of samples and Ward's minimum variance rule for the merging of cluster branches. The correlation coefficient between each probe set and the *IFNG* gene (represented by the probe set 1425947_at) was also computed using the log 2 expression values. Although we computed the correlation coefficient for all probe sets, we report only those probe sets with an average expression above 25. Probe sets that did not meet this criterion were considered absent. The differential expression of genes upon IFN-γ treatment was assessed in immortalized primary cells. We report the magnitude of the differential expression in the conditions "average IFN-γ treated" and "untreated" as the average difference of the log 2 values in the two conditions. The differential expression is only reported for probe sets that were declared present (average expression above 25) in one of the two conditions.

Neutrophil quantification by myeloperoxidase activity assay

The myeloperoxidase assay was adapted from Mota et al. (36). One-sixth of every stomach was homogenized in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyl trimethyl ammonium bromide (H5882; Sigma-Aldrich). Homogenates were then subjected to four cycles of freezing in liquid nitrogen and thawing at room temperature. After a subsequent centrifugation step at 4°C for 20 min, the supernatants were collected and their protein content was quantified. Forty micrograms of total protein was analyzed for myeloperoxidase activity in a final reaction volume of 300 μl containing 65 mM potassium phosphate/0.5% hexadecyl trimethyl ammonium bromide, 1.6 mM tetramethylbenzidine (T2885; Sigma-Aldrich), and 0.3 mM H_2O_2. Absorbance was measured photometrically at 655 nm.

Statistics

Most statistical analysis was performed by Student's *t* test or Mann-Whitney *U* test using GraphPad Prism software, as indicated in the figure legends. For computing the strength and the significance of the statistical dependence of colonization levels and IFN-γ expression, we used Pearson's correlation coefficient. Calculating the statistical dependence of IFN-γ expression on each of the histological categorizations (chronic inflammation, atrophy, metaplasia, hyperplasia) required using a test for conditional independence described by Hothorn et al. (37).

[4] Abbreviations used in this paper: PCNA, proliferating cell nuclear Ag; IP-10, IFN-γ-inducible protein 10.

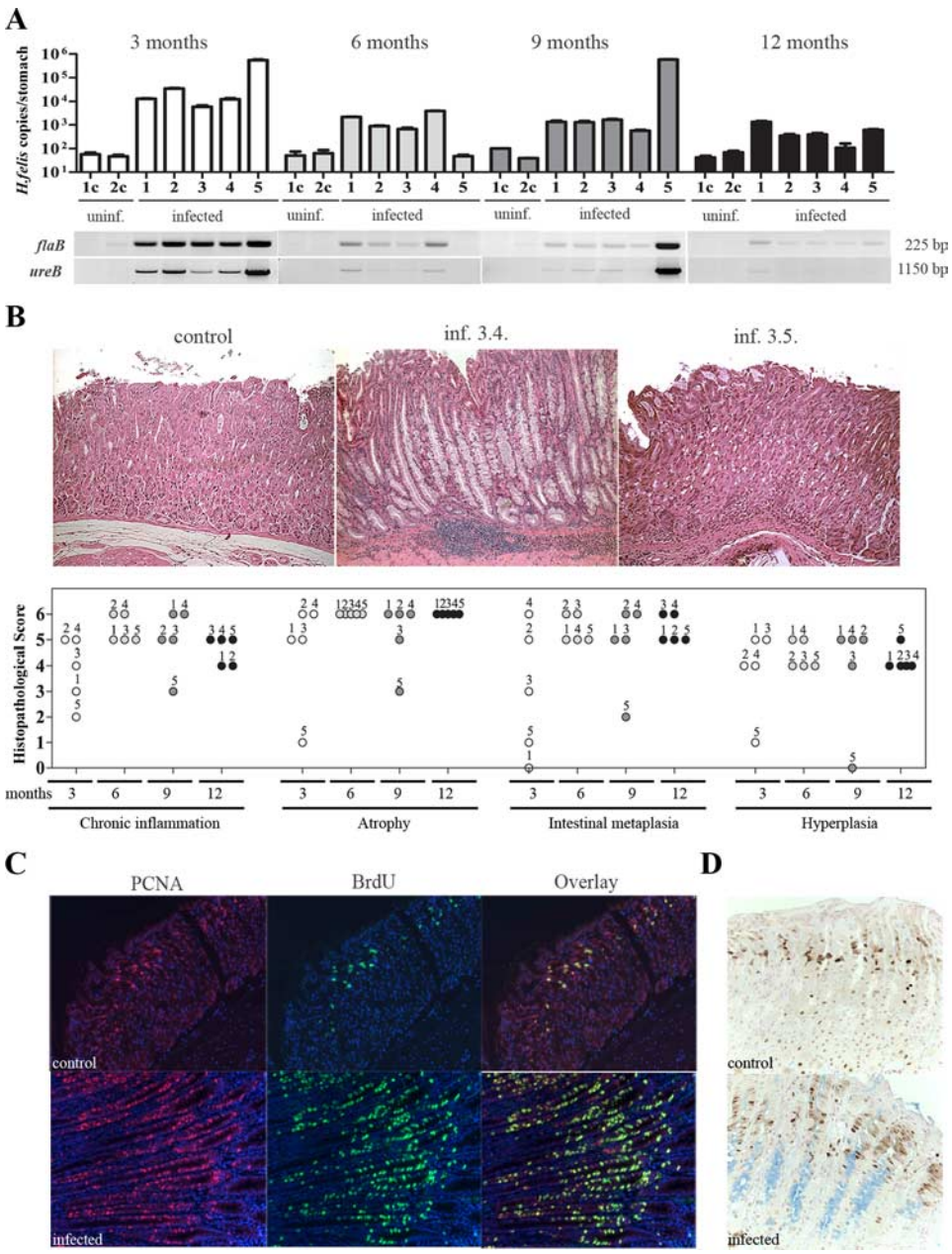


FIGURE 1. Male C57BL/6 mice infected with *H. felis* for 3, 6, 9, and 12 mo develop gastric inflammation and preneoplastic pathology. *A*, Quantitative assessment of *H. felis* colonization by PCR directed against the *flaB* and *ureB* genes using whole stomach genomic DNA as template. Real-time results for the *flaB* PCR are shown in triplicate with errors bars above the gel photographs for both PCRs for all five infected and two uninfected mice per time point. The color code indicates the duration of infection. *B*, Histopathological analysis of gastric tissue. H&E-stained sections are shown for a representative uninfected (*left panel*) and two 3-mo infected mice (*middle* and *right panels*). Histopathological scores are shown on a scale of 0–6 for four parameters: chronic inflammation, atrophy, intestinal metaplasia, and hyperplasia. Every mouse was scored; scores for all five infected mice per time point are shown along with the mouse identification. Uninfected control mice were scored as well; all scores were 0 (data not shown). The color code is the same as in *A*. *C*, *H. felis* infection induces epithelial hyperplasia. BrdU- and PCNA-stained sections from the corpus of two representative control and infected mice are shown. In an uninfected stomach, actively proliferating progenitor cells populate a narrow band in the isthmus; in contrast, in a chronically infected stomach, this proliferative zone can expand to include the entire gland. *D*, PCNA and Alcian blue costaining of an uninfected control and an infected mouse reveals localization of hyperplastic cells (brown nuclei) in the luminal half of the gland, whereas metaplastic cells (blue) are found at the base of the gland.

Results

Chronic infection of C57BL/6 mice with H. felis *results in gastric inflammation and preneoplastic pathology*

To assess the effects of chronic *H. felis* infection of male C57BL/6 mice on the gastric mucosa, 20 mice were infected and monitored in a temporal manner with respect to colonization levels and gastric pathology. Since *H. felis* does not readily form colonies, colonization was examined by visual inspection of Giemsa-stained sections and by PCR using two different sets of *H. felis*-specific primers targeting the *flaB* and *ureB* genes. The primer combination amplifying parts of the *flaB* gene was further used for quantitative PCR to determine the absolute number of *H. felis* bacteria per stomach as previously described (33). Quantitative histopathological analysis was performed on H&E-, Giemsa-, periodic acid-Schiff-, and Alcian blue-stained tissue sections based on the features described in the updated Sydney classification (34), with a quantitative scoring system as devised by Chen et al. (35). At 3 mo after infection, the bacteria were readily detectable in histological sections (data not shown) and by PCR (Fig. 1*A*), with bacterial densities ranging from 10^4 to 10^6 bacteria per stomach. Extensive infiltration of the infected mucosa and submucosa by various immune cell populations was observed (H&E staining, Fig. 1*B*, *top middle*, *right*, and *bottom panels*), which is consistent with a diagnosis of chronic active gastritis. H&E staining further revealed cells that histologically resemble intestinal goblet cells (Fig. 1*B, top middle panel*) in several mice at this early time point; these cells are strongly stained also by Alcian blue (which stains neutral, sulfated mucopolysaccharides; Fig. 1*D*). The appearance of intestinal-like cells is accompanied by the partial or complete loss of terminally differentiated gastric cells of the parietal and chief cell lineages, thereby fulfilling the criteria for the diagnosis of gastric atrophy or atrophic gastritis (Fig. 1*B, top middle* and *bottom panels*). Alcian blue-positive metaplasia is mostly found in the proximal third of the corpus, i.e., close to the forestomach junction, at this early time point; the distal corpus is populated by a rapidly proliferating, PCNA-positive and BrdU-incorporating cell type

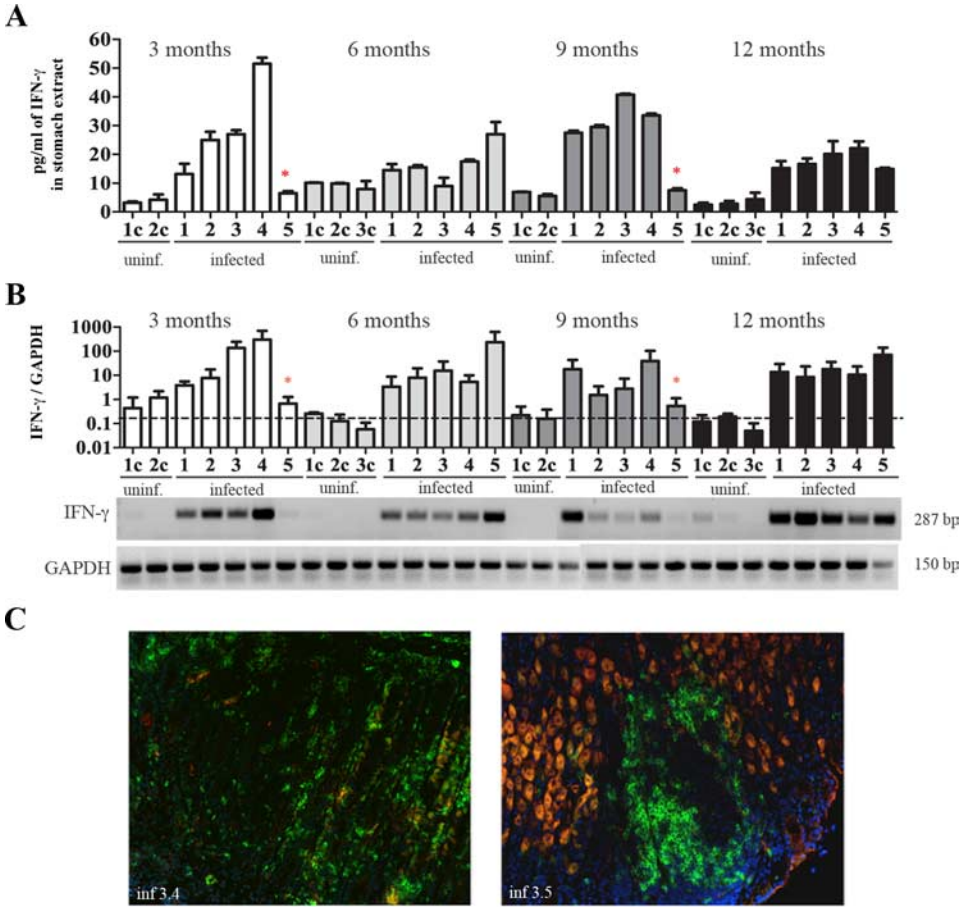


FIGURE 2. Gastric IFN-γ production and $CD4^+$ T cell infiltration patterns are indicative of colonization levels and pathology. *A*, IFN-γ ELISA revealing gastric IFN-γ production in uninfected (uninf) and infected mice 3, 6, 9, and 12 mo after infection. The animals shown are the same as in Fig. 1. *B*, Gastric IFN-γ transcript levels as determined by conventional (*bottom panels*) and real-time (*upper panels*) RT-PCR are shown for each animal of the time course, with GAPDH levels serving as loading controls and for normalization of real-time IFN-γ expression. Mice marked with a red asterisk have lower IFN-γ transcript levels than the other members of their groups. The color code is the same in *A* and *B* and also the same as in Fig. 1. *C*, Mice with severe pathology are characterized by $CD4^+$ T cells (in green) infiltrating diffusely into the lamina propria (e.g., mouse 3.4., *left panel*). In contrast, a mouse with little or no epithelial changes displays clusters of $CD4^+$ T cells that resemble lymphoid follicles (mouse 3.5., *right panel*). The absence of parietal cells (orange) indicates gastric atrophy.

(Fig. 1*C, lower panel*) that morphologically resembles the progenitor of surface mucus (pit) cells. In an uninfected age-matched stomach, PCNA-positive cells (i.e., gastric stem cells or their immediate progeny) reside exclusively in a narrow band of mucosa termed the isthmus, at a density of two to four cells per gland (Fig. 1*C, upper panel*). In contrast, in chronically infected mice, the entire gland is sometimes found to be PCNA and BrdU positive from the base to the tip. The overrepresentation of this PCNA-positive, rapidly cycling progenitor cell is consistent with a diagnosis of compensatory epithelial hyperproliferation, also termed foveolar hyperplasia or mucus pit cell hyperplasia. Overall, at 3 mo after infection, the predominant histopathological lesions observed are moderate to marked atrophy, moderate epithelial hyperplasia, and evidence of beginning intestinal metaplasia in the proximal part of the corpus (Fig. 1*B, lower panel*), all of which are recognized precursor lesions of gastric cancer. Although metaplasia and hyperplasia usually affect different regions of the corpus within the same infected animal, both types of lesions can sometimes be found in the same gland (Fig. 1*D, lower panel*). In these cases, their localization in the gland relative to each other suggests that hyperplastic cells arise from progenitors migrating from the isthmus toward the stomach lumen (i.e., surface mucus cell progenitors), whereas metaplastic cells arise from progenitors of those lineages that differentiate as they migrate toward the base of the gland, i.e., parietal or chief cells.

At the later time points of the study (6, 9, and 12 mo postinfection), bacterial colonization decreased progressively in the majority of mice (Fig. 1*A*). This decrease in bacterial densities was accompanied by a slight overall increase in the surface area of the corpus affected by marked pathology (Fig. 1*B, lower panel*), but progression to carcinoma was not observed in the time frame of our study.

Notably, however, we found that in two of the four time points, several animals did not fit into the overall pattern (i.e., animals 3.5. and 9.5., Fig. 1, *A* and *B*). These mice were colonized at very high levels ($>10^5$ bacteria/stomach), but had minor or no evidence of preneoplastic epithelial changes (Fig. 1*B, bottom panel*). Atrophy was not detected; instead, the gastric mucosa of the mice was characterized by aggregates of lymphocytes confined to few, very restricted areas. The surrounding mucosa was normal with respect to its cellular composition. These exceptional mice therefore differed substantially from their littermates in that they were protected from gastric pathology despite harboring high bacterial loads.

The two types of responses to Helicobacter *infection differ with respect to IFN-γ production*

Because Th1-polarized T cells and their signature cytokine IFN-γ had been implicated before in *H. pylori*-induced gastritis (38), we hypothesized that this cytokine might be differentially regulated in mice differing with respect to gastric pathology. We analyzed local gastric IFN-γ expression in all of the mice of the time course shown in Fig. 1 by ELISA (Fig. 2*A*) as well as conventional and real-time RT-PCR (Fig. 2*B*). Strong IFN-γ production was detected only in those mice that had successfully reduced bacterial colonization and had significant associated immunopathology. In contrast, the exceptional mice 3.5 and 9.5 of the time course study (marked by red asterisks, Fig. 2, *A* and *B*) failed to mount a measurable IFN-γ response, as determined at both the RNA and protein levels. The other striking difference between the two groups of

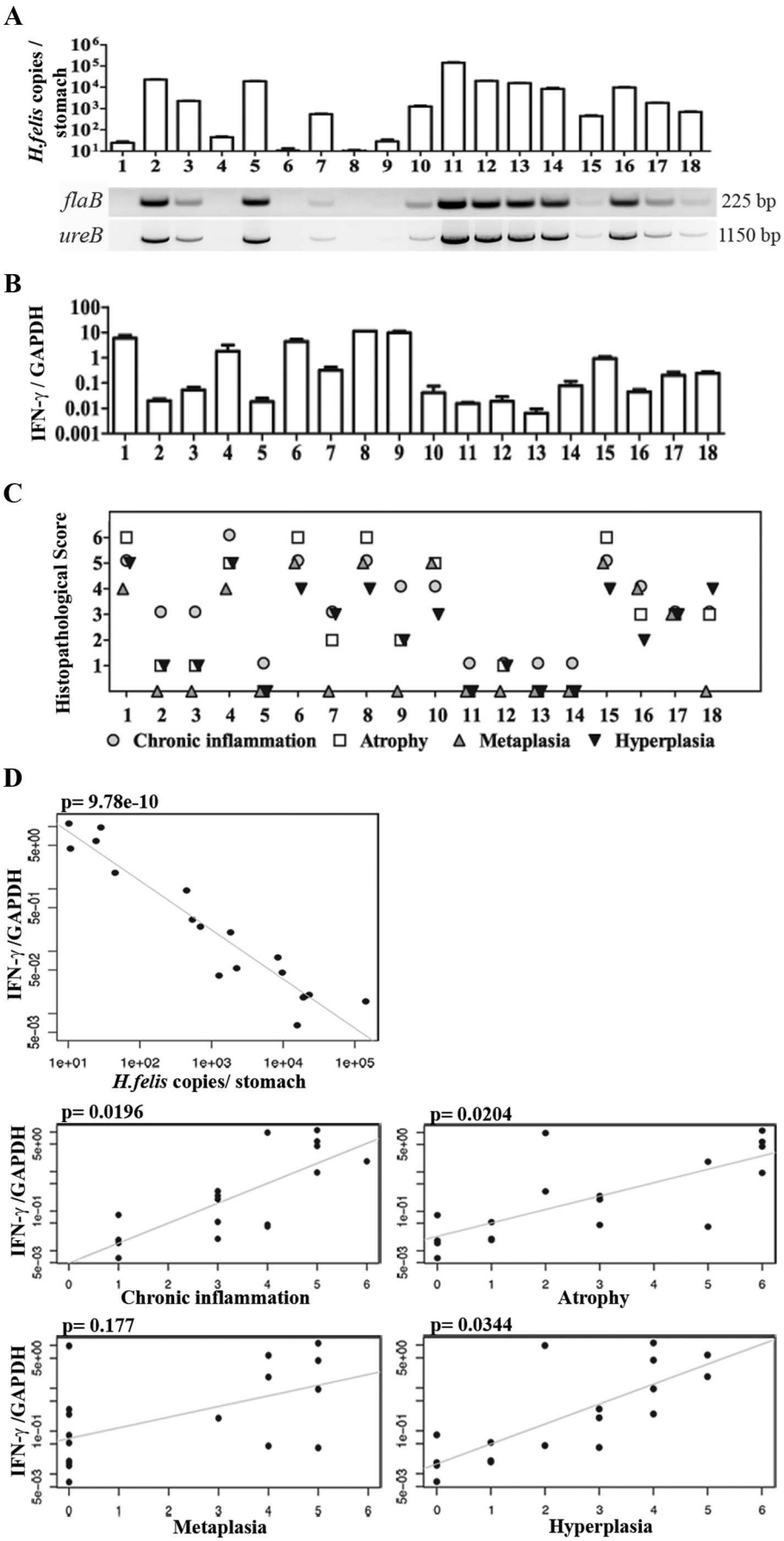


FIGURE 3. Gastric IFN-γ production is inversely correlated with *Helicobacter* colonization and determines gastric preneoplastic pathology. Eighteen mice were infected with *H. felis* for 3 mo. *A*, *H. felis* colonization levels as determined by *flaB* real-time PCR are shown in triplicate with errors bars above the gel photographs for *flaB* and *ureB* PCR for all 18 mice (numbered). *B*, Normalized gastric IFN-γ expression as determined by real-time PCR is shown for the same mice as in *A*. *C*, Histopathology scores for the four parameters indicated in the legend are shown for all 18 mice. *D*, Normalized IFN-γ expression is plotted as a function of *H. felis* colonization (*top panel*) as well as the scores for the four parameters (*lower panels*; each dot represents one mouse). Colonization and IFN-γ expression are anticorrelated with a Pearson's correlation coefficient of -0.95 and an associated p value of 9.78^{-10}. The p values indicating the significance of the correlation of IFN-γ expression with the histopathological parameters were calculated as described in *Materials and Methods* and are also shown.

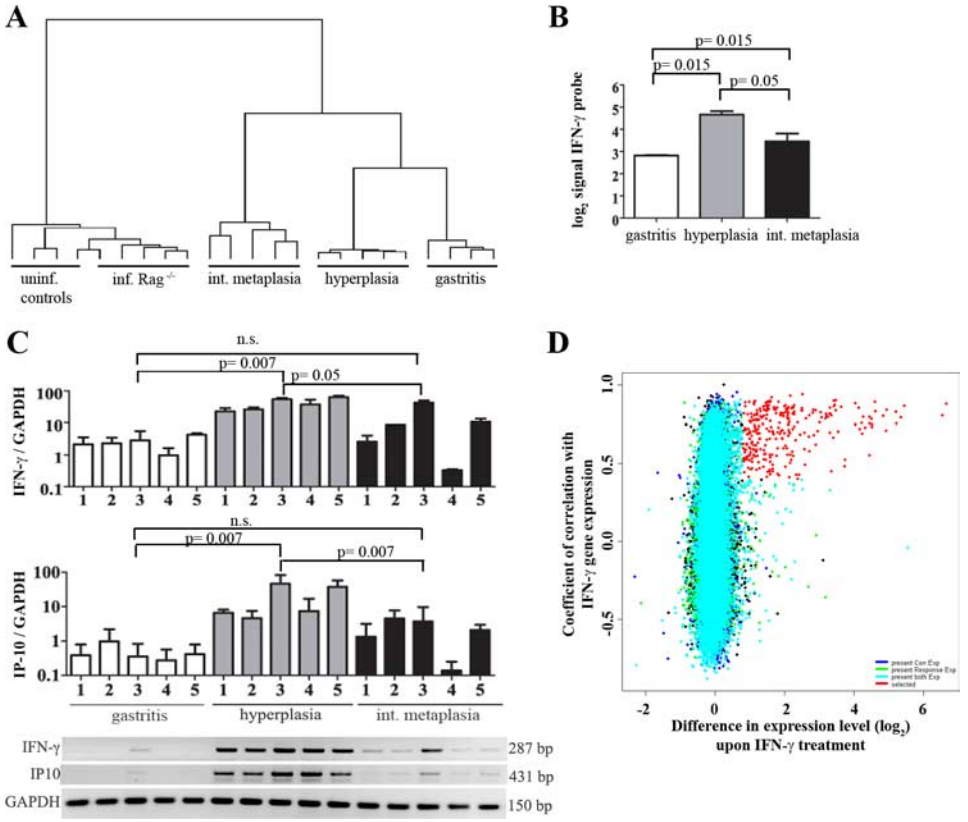


FIGURE 4. IFN-γ expression is high in murine hyperplastic mucosa, but not in metaplastic mucosa or mucosa that is inflamed, but has not undergone epithelial changes. *A*, Gene expression profiling segregates samples representing uninfected (uninf) gastric mucosa from those of infected (inf) mucosa displaying gastritis and from those of infected mucosa with a predominantly hyperplastic or metaplastic phenotype. RNA was extracted from the scraped mucosa of mice that displayed uniform pathology of either of the three types, reverse transcribed, and hybridized to Affymetrix Genechips. Hierarchical clustering of the samples was performed using Rosetta Resolver software. Sample identities (four to five mice per group) are noted below the respective branches of the dendrogram tree. *B*, Averages of signals corresponding to the IFN-γ probe on the array, as calculated for every histopathologically defined group of five infected mice. Error bars and *p* values (Mann-Whitney *U* test) are also shown. *C*, Conventional and real-time RT-PCR of IFN-γ-, IP-10-, and GAPDH-specific transcripts performed on five infected mice per histological group as shown in *A* and *B*. Triplicates were measured and error bars are shown. The *p* values were calculated by the Mann-Whitney *U* test. The color code is the same in *B* and *C*. *D*, All genes present on the array were assessed in terms of their 1) regulation by IFN-γ in an immortalized murine gastric epithelial cell line and 2) their correlation of expression with IFN-γ across the arrays shown in *A*. Differential expression upon IFN-γ treatment is plotted on the x-axis in $\log_2$ scale and the coefficient of correlation with IFN-γ expression in vivo is plotted on the y-axis. Genes called present in the in vitro study (IFN-γ treatment of cells) are shown in green and genes called present in the murine mucosa (comparison of mice with various degrees of pathology, see *A*) are shown in dark blue. Genes called present in both data sets are shown in turquoise. Those genes for which the correlation coefficient was >0.4 and the log 2 ratio was >0.8 were selected and are shown in red, which indicates their induction upon IFN-γ treatment in vitro and their correlation with IFN-γ expression in vivo. This subset of genes therefore is likely induced by IFN-γ in vivo. All arrays hybridized in the course of the two studies are publicly accessible on the National Center of Biotechnology Information GEO web site (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13873). The *p* value indicating that the high-response probes are overrepresented in the high-correlation probes (as computed by Fisher's exact test) is 3.45×10^{-67}.

mice was their pattern of gastric infiltration by $CD4^+$ T cells (Fig. 2*C*). Whereas these cells were found to be confined to large mucosal aggregates in highly colonized mice (Fig. 2*C*, *right panel*), they were more diffusely distributed in the group of mice characterized by low bacterial counts and severe epithelial pathology (Fig. 2*C*, *left panel*). In this group, the T cells infiltrated the entire lamina propria from the base to the tip of the gland in small groups or as single cells, suggesting that this type of $CD4^+$ T cell mediates bacterial clearance and induces epithelial changes. Parietal cells (in orange, Fig. 2*C*) were almost completely absent from the stomachs infiltrated in this manner, but are found in normal numbers in the highly colonized mice. Interestingly, the effects of diffusely infiltrating T cells often are extremely localized, i.e., hyperplasia and metaplasia are found only in glands directly adjacent to these infiltrates (data not shown). Overall, the differences between mice with or without pathology suggest that a soluble factor secreted by lamina propria-infiltrating T cells, possibly IFN-γ, induces the preneoplastic changes characteristic of *H. felis*- infected C57BL/6 mice.

To assess the association of IFN-γ expression and gastric pathology on the one hand and the possible inverse relationship of IFN-γ levels with *Helicobacter* colonization on the other in a more representative group of animals, we analyzed 18 additional C57BL/6 mice infected with *H. felis* for 3 mo with respect to colonization (Fig. 3*A*), IFN-γ expression (Fig. 3*B*), and gastric histopathology (Fig. 3*C*). Interestingly, we found that colonization and IFN-γ expression were indeed anticorrelated, with a Pearson's correlation coefficient of −0.95 and an associated *p* value of 9.78^{-10} (Fig. 3*D*). IFN-γ levels were further significantly associated with chronic inflammation, atrophy, and epithelial hyperplasia (*p* values of 0.0196, 0.0204, and 0.0344) and less closely linked to the appearance of metaplasia ($p = 0.177$; Fig. 3*D*). From these calculations, we conclude that local IFN-γ expression is indeed a useful indicator of a successful reduction of bacterial burden, as

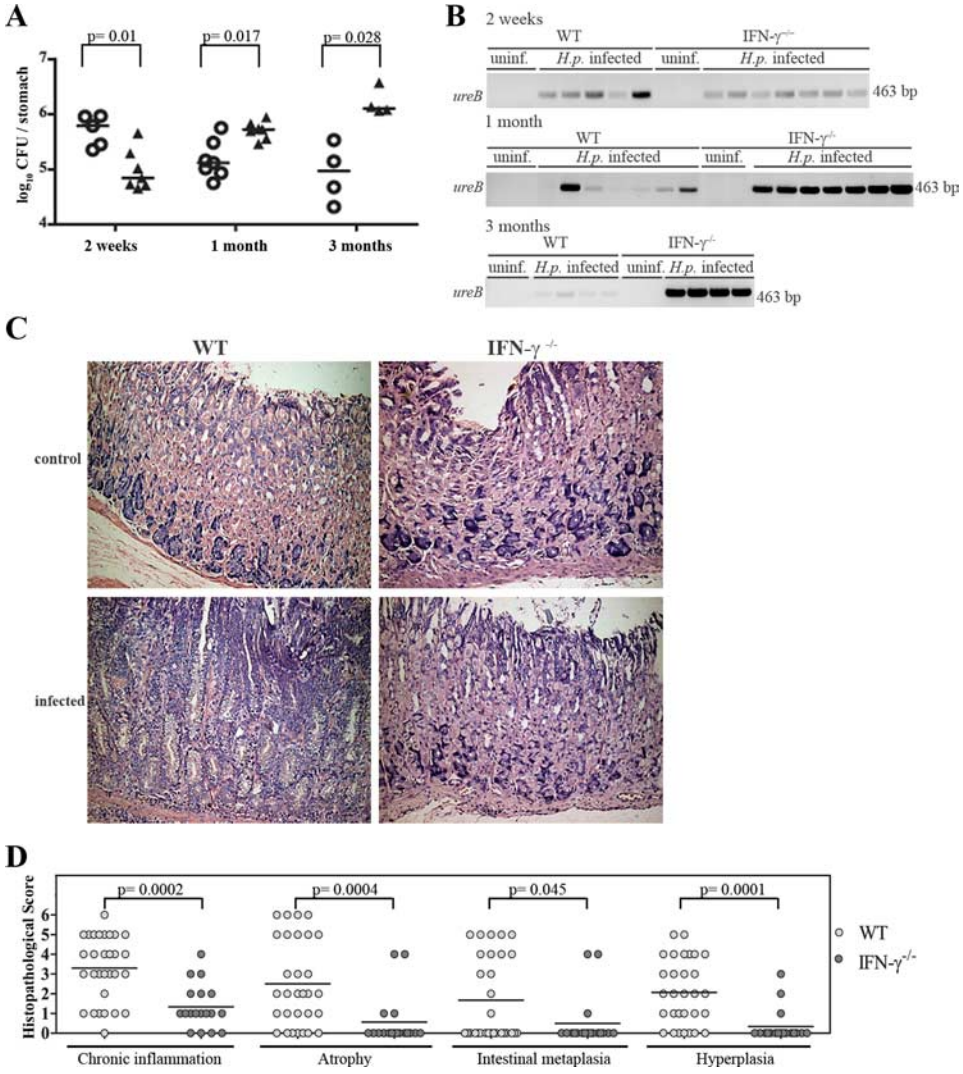


FIGURE 5. IFN-γ is crucial for the efficient reduction of *H. pylori* burden and for the development of *H. felis*-induced premalignant lesions. *A* and *B*, Wild-type (WT) C57BL/6 mice (○) and IFN-$\gamma^{-/-}$ Bl6 mice (△; four to seven mice per group) were infected with *H. pylori* SS1. Colonization was assessed by colony counting (*A*) and *H. pylori*-specific PCR using *ureB*-directed primers (*B*) at 2 wk, 1 mo, and 3 mo after infection. Counts and PCR results are shown along with *p* values indicating significance of the findings as determined by the Mann-Whitney *U* test (*A*). *C*, Giemsa-stained sections are shown for representative C57BL/6 wild-type and IFN-$\gamma^{-/-}$ mice infected for 3 mo with *H. felis* along with uninfected controls. *D*, Histopathology scores for four histopathological parameters (as indicated) for a total of 30 wild-type and 18 IFN-$\gamma^{-/-}$ mice included in three independent studies. Values of *p* as calculated by Student's *t* test indicate the statistical significance of the differences. Thin bars in *A* and *D* indicate the means.

well as a valid predictor of gastric premalignant changes triggered by *Helicobacter* infection.

Gene expression profiling reveals distinct transcriptional signatures in nonatrophic inflamed vs predominantly hyperplastic vs metaplastic gastric mucosa

To assess whether the histopathological differences between the two groups of mice responding differentially to *Helicobacter* infection translate into transcriptionally evident differences, we performed gene expression profiling using Affymetrix Genechips. RNA was purified from scraped mucosal samples of an independent set of *H. felis*-infected mice that were chosen because they had developed uniform pathology of either of the three following types rather than a mixture of all three: 1) symptoms of gastritis, but no epithelial changes; 2) atrophic gastritis accompanied by epithelial hyperplasia only; or 3) atrophic gastritis accompanied by intestinal metaplasia only. An uninfected control group was also included in the analysis, as were two groups of mice that lacked mature T and B cells due to a deletion mutation in the *rag-1* gene (Rag-$1^{-/-}$) and that were either experimentally infected or served as Rag-$1^{-/-}$ uninfected controls. Infected Rag-$1^{-/-}$ mice showed no signs of gastritis despite high colonization levels. An unsupervised hierarchical clustering approach, which deliberately does not take into account the histopathological information we had on the mice, revealed the segregation of inflamed, but nonatrophic (i.e., no epithelial changes) samples from those characterized by hyperplasia or metaplasia (Fig. 4*A*). Interestingly, the latter two were also significantly different from each other to drive their segregation into two distinct branches of the dendrogram tree. All control samples are found in a separate branch. These cluster along with the samples of infected as well as uninfected Rag-$1^{-/-}$ mice, reflecting the complete lack of gastric inflammation in this genetic background (Fig. 4*A*). Indeed, the transcriptional changes between infected and uninfected Rag-$1^{-/-}$ mice affected only a small number of genes and were thus insufficient to drive clustering into distinct groups. In conclusion, the clustering of histologically similar samples into distinct branches of the dendrogram tree suggests distinct pathogenetic pathways for hyperplastic and metaplastic lineages and provides transcriptional signatures of these lesions (publicly accessible under http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE13873) that can now be mined for clues to their cellular and pathogenetic origin.

Strikingly, the expression of IFN-γ was strongest in the hyperplastic group as judged by the signal intensities of the corresponding probe sets on the array (Fig. 4*B*). As expected, nonatrophic gastritis samples did not express IFN-γ (Fig. 4*B*), confirming our previous observations (Figs. 2 and 3). Interestingly, the metaplastic samples also showed significantly less IFN-γ production than hyperplastic samples, suggesting that hyperplasia is the dominant

lesion induced by local IFN-γ production (Fig. 4*B*). This was confirmed by IFN-γ-specific conventional RT-PCR as well as real-time RT-PCR performed on the same set of animals (Fig. 4*C*). Real-time and conventional RT-PCR designed to amplify a known target gene of the IFN-γ signaling pathway, the chemokine IP-10 (also known under the name CXCL-10) was also performed and revealed a virtually identical pattern of expression (Fig. 4*C*). In summary, we conclude from the two independent experiments described in Figs. 3 and 4 that local IFN-γ production is significantly associated with the induction of hyperplastic, but not metaplastic lesions as a result of the infection, again suggesting that the pathogenetic origins of these two premalignant lineages are probably distinct.

Aside from the known IFN-γ target IP-10, the expression patterns of numerous genes correlate with the expression of IFN-γ in our data set (for a list of all genes represented on the array and their correlation with IFN-γ expression, see supplemental Table I[5]). In an effort to identify genes that are not only correlated, but induced directly by this cytokine, we generated an immortalized cell line from primary murine gastric epithelial cells and treated these cells with increasing concentrations of recombinant murine IFN-γ for 24 h. mRNA extracted from these cells was then subjected to transcriptional profiling analysis using Genechips. All genes on the array were then analyzed for their correlation with IFN-γ on the one hand (first data set, dark blue spots in Fig. 4*D*) and their induction *by* IFN-γ on the other (second data set, green spots in Fig. 4*D*; turquoise spots indicate representation in both data sets). Interestingly, the vast majority of all 286 genes found to be strongly induced by IFN-γ in vitro were also correlated with IFN-γ in vivo (red spots), suggesting that these genes are induced by IFN-γ in vivo (for a list of these genes, see supplemental Table II).

IFN-γ$^{-/-}$ mice are colonized more heavily and have less severe Helicobacter*-induced pathology than wild-type mice*

Since IFN-γ production is correlated with both a reduction in bacterial burden and induction of preneoplastic epithelial changes, we assessed both in an IFN-γ-deficient background. For quantification of bacterial colonization, C57BL/6 wild-type and IFN-γ$^{-/-}$ mice were infected with *H. pylori* strain SS1, a mouse-adapted patient isolate that readily forms colonies and can also be subjected to *H. pylori*-specific semiquantitative PCR using a primer combination amplifying the urease subunit B gene (*ureB*) from gastric genomic DNA. IFN-γ$^{-/-}$ mice were colonized more heavily than wild type at 4 and 12 wk after infection, and this difference was significant at both time points (Fig. 5, *A* and *B*). In contrast, the colonization levels were reversed at 2 wk after infection, suggesting that the defect of the IFN-γ$^{-/-}$ strain with respect to clearance becomes evident only after an adaptive immune response starts to take effect. These findings suggest that IFN-γ is critical for the control of *Helicobacter* infection during the adaptive phase of the immune response.

Interestingly, IFN-γ$^{-/-}$ mice were further protected from preneoplastic changes induced by 3 mo of *H. felis* infection in three independent studies comparing them to wild-type mice (Fig. 5, *C* and *D*). They were significantly less likely than wild- type mice to develop gastritis, atrophy, hyperplasia, or metaplasia (with *p* values of 0.0002, 0.0004, 0.0001, and 0.045, respectively). This observation suggests that the preneoplastic phenotype induced by *Helicobacter* infection in the C57BL/6 background depends on IFN-γ production.

[5] The online version of this article contains supplemental material.

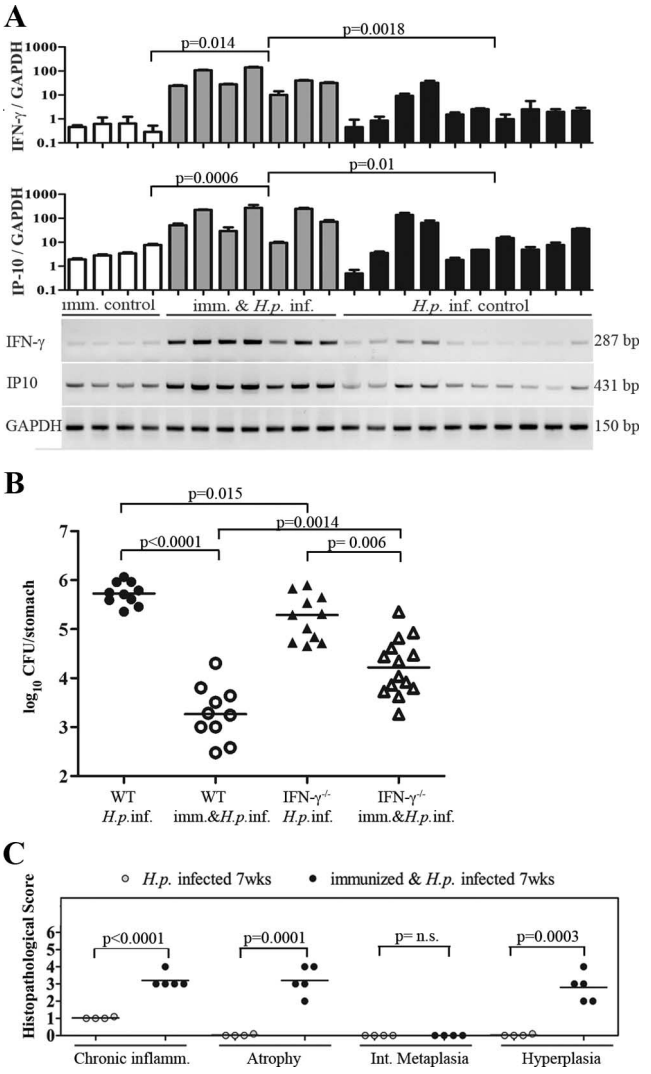


FIGURE 6. IFN-γ is induced upon and required for efficient vaccination against *H. pylori* (H.p.). Vaccinees were immunized four times in weekly intervals with 1 mg of *H. pylori* sonicate in conjunction with 10 μg of cholera toxin and challenged with *H. pylori* strain SS1 2 wk after the last immunization. Colonization was determined 2 wk (*A* and *B*) or 7 wk (*C*) after challenge. *A*, IFN-γ and IP-10 levels were assessed by conventional (*lower panels*) and real-time (*upper panels*) RT-PCR for a group of immunized (imm&H.p.inf.) and challenged (inf.) wild-type (WT) C57BL/6 mice, a group of immunized, but uninfected (imm. control) mice and a group of control-infected mice (inf. control), respectively. GAPDH levels served as loading controls and for normalization of IFN-γ expression. *B*, Colony counts of 10 wild-type and 14 IFN-γ$^{-/-}$ mice (imm.&H.p.inf.) that were immunized as described above. Ten additional wild-type and 11 additional IFN-γ$^{-/-}$ mice served as infection controls (inf.). The *p* values were calculated using the Mann-Whitney *U* test. Thin bars indicate the medians. *C*, Histopathology scores of five immunized wild-type C57BL/6 animals 7 wk after challenge with SS1 compared with four mice that were infected without prior vaccination for 7 wk. The *p* values indicate significant differences for three of the parameters scored; metaplasia was not observed in any of the mice. Thin bars indicate the means. n.s., Not significant.

IFN-γ$^{-/-}$ mice are less protected than wild-type mice upon immunization with a whole cell sonicate H. pylori *vaccine*

Helicobacter cannot be cleared spontaneously in mice or humans. We were therefore interested in testing the relevance of IFN-γ in a true clearance model. For this purpose, we used a vaccine regimen consisting of four consecutive weekly doses of 1 mg of *H.*

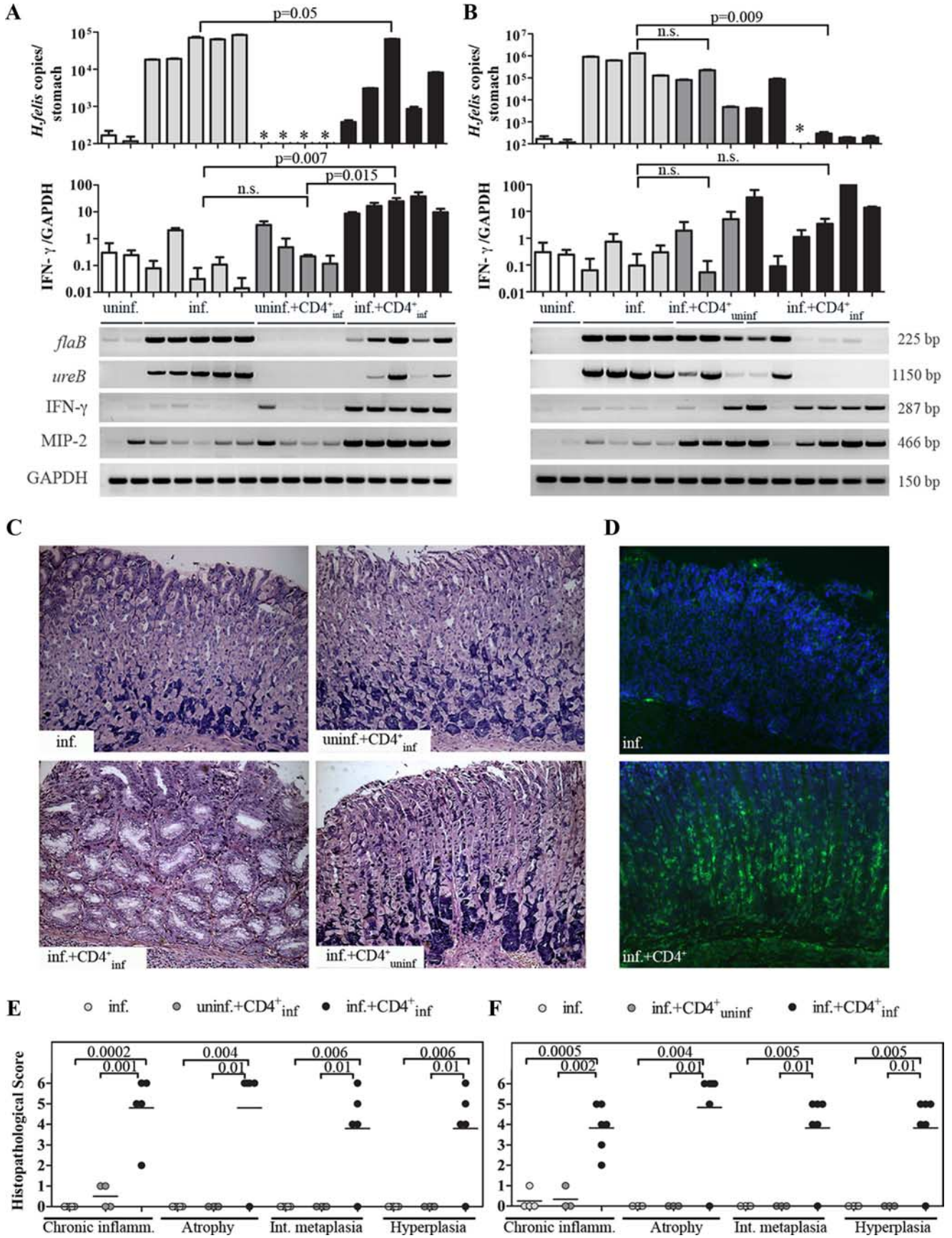


FIGURE 7. Adoptive transfer of $CD4^+CD25^-$ T cells into immunodeficient mice induces *Helicobacter* clearance, strong local IFN-γ and MIP-2 production, and gastric preneoplastic lesions. *A*, Immunomagnetically purified $CD4^+CD25^-$ T cells from the spleens of *H. felis*-infected wild-type donors were adoptively transferred into 6-wk-old Rag-$1^{-/-}$ mice (300,000 cells/mouse), which were subsequently infected with *H. felis* (inf. + $CD4^+_{inf}$) or remained uninfected (uninf. + $CD4^+_{inf}$) for the duration of the experiment (1 mo). gDNA was isolated from every stomach and used for conventional PCR

pylori SS1 sonicate plus 10 μg of cholera toxin followed by challenge with the same strain 2 wk after administration of the last dose. This regimen leads to a significant reduction in bacterial burden by two orders of magnitude, from 10^5 to 10^6 CFU/stomach to 10^3–10^4 CFU/stomach (Fig. 6*B*) in most wild-type mice. Immunized/challenged wild-type mice produce IFN-γ at significantly higher levels than immunized controls or mice from a control group that was infected without prior immunization, as assessed by conventional and real-time RT-PCR (Fig. 6*A*). This is further reflected in the transcript levels of IP-10, a downstream mediator of IFN-γ (Fig. 6*A*). In contrast to the successful clearance of *H. pylori* SS1 from stomachs of immunized wild-type mice, IFN-$\gamma^{-/-}$ mice receiving the same vaccine were significantly less capable of clearing the infection (Fig. 6*B*; $p = 0.0014$), implying that a strong IFN-γ response is crucial for vaccination-induced protection.

IFN-γ is known to act on numerous immune and nonimmune cell populations. Best understood is its effect on macrophages, which are rendered highly bactericidal and are induced to efficiently present Ag in the context of MHC class II upon exposure to IFN-γ (39). We assessed the role of macrophages on clearance in our vaccine model by depleting them with liposome-encapsulated chlodronate (a bisphosphonate toxin; data not shown) during the challenge phase of our immunization protocol. Despite efficient depletion of macrophages from the spleen under these circumstances (40), we saw no negative effects on clearance, suggesting that macrophages are dispensable for the effector phase of *Helicobacter* clearance (data not shown).

Vaccination with *H. pylori* sonicate and cholera toxin reduces the bacterial burden, but does not eliminate the infection entirely (Fig. 6*B*). The residual colonization is of concern, as it may foster a chronic immune response in the stomach that is more virulent than the response to the natural infection. To assess the pathology that is triggered by a vaccine-induced response over and above what is induced by infection with the same strain alone, vaccinated/challenged mice were compared with an infected-only group 7 wk after infection (Fig. 6*C*). The vaccinated group scored significantly higher with respect to inflammation, atrophy, and hyperplasia, while intestinal metaplasia was not detected at this point in time (Fig. 6*C*). Although vaccine-induced gastric pathology may be transient at least with a weakly immunogenic challenge strain such as SS1 (as has been suggested by Sutton et al. (41)), these results imply that sterile immunity should be the goal of *Helicobacter* vaccine development efforts.

$CD4^+CD25^-$ effector T cells control Helicobacter *infection and rapidly induce preneoplastic epithelial changes in immunodeficient recipients*

Multiple cell types are known to produce IFN-γ during the early, innate, and the adaptive phases of the immune response to invading pathogens. NK cells have been shown to be an important early source of IFN-γ for example during *Legionella pneumophila* infection (42). NKT cells as well as subsets of $CD4^+$ and $CD8^+$ T cells are possible sources of IFN-γ during the adaptive phase of the immune response. To assess the role of NK and NKT cells during *H. pylori* infection, we depleted them in vivo using a mAb directed against their shared NK1.1 surface marker in the course of a 12-day experimental infection. Alternatively, NKT cells were selectively activated in a separate group of mice by i.p. injection of α-galactosylceramide, a known ligand of the semi-invariant Vα14 TCR that is recognized in the context of CD1d molecules on APCs. Neither treatment influenced colonization levels in a significant manner (data not shown), ruling out an important contribution of the two cell types in this experimental infection model.

To assess the functional contribution of $CD4^+$ T cells to *Helicobacter* control and to *Helicobacter*-induced immunopathology, we used an adoptive transfer model of purified $CD4^+CD25^-$ T cells into immunodeficient hosts. Rag-$1^{-/-}$ (mature T and B cell-deficient) and TCR-$\beta^{-/-}$ (lacking the TCR-α/β^+ subset of T cells) mice were used interchangeably as recipients in multiple independent experiments and produced identical results. Both strains are essentially protected from *Helicobacter*-induced gastric pathology due to their defect in mounting a proper T cell response to the infection (Fig. 7*C*, inf.). Both are colonized at higher levels than wild-type mice (data not shown). Adoptive transfer of ~300,000 $CD4^+CD25^-$ T cells isolated from a *H. felis*-infected wild-type donor spleen induced a significant reduction in bacterial burden ($p = 0.05$ and 0.009; compare light gray and black bars showing quantitative *flaB* PCR performed on gastric genomic DNA; Fig. 7, *A* and *B*) as early as 4 wk after infection compared with infected controls that had not received cells. Clearance was accompanied by strong gastric IFN-γ responses (assessed by conventional and real-time RT-PCR; Fig. 7, *A* and *B*, compare light gray and black

directed against the *flaB* and *ureB* genes of *H. felis* (*lower panels*) as well as quantitative *flaB* PCR (*top panel*) to determine colonization levels. IFN-γ, MIP-2, and GAPDH transcripts were assessed by conventional RT-PCR (*lower panels*); IFN-γ was also quantified by real-time PCR (*middle panel*). Uninfected and *H. felis*-infected mice that did not receive cells are shown as controls. Values of *p* as calculated by Mann-Whitney *U* test are shown for all quantitative comparisons; n.s., not significant. Asterisks indicate values that were below the detection limit. *B*, In this experiment, the setup was identical to that described in *A*, except that $CD4^+CD25^-$ T cells were isolated from infected or uninfected donors, respectively (inf. + $CD4^+_{inf}$ or inf. + $CD4^+_{uninf}$) and all recipients were infected upon transfer. The duration of this experiment was 3 mo from the day of adoptive transfer. Again, uninfected and *H. felis*-infected mice that did not receive cells served as controls. *C*, Representative Giemsa-stained sections are shown from a mouse that was *H. felis*-infected (inf.), but did not receive cells, a mouse that received cells, but remained uninfected (uninf. + $CD4^+_{inf}$), a mouse that received cells from an infected donor and was infected (inf. + $CD4^+_{inf}$), and a mouse that received cells from an uninfected donor and was infected (inf. + $CD4^+_{uninf}$). Only the mouse that received cells from an infected donor and was infected itself developed gastric pathology such as intestinal metaplasia. A quantitative histopathological analysis of all mice included in the studies described in *A* and *B* is shown in *E* and *F*, respectively. *D*, Immunofluorescent staining of the CD4 surface marker in cryosections of a representative infected Rag-$1^{-/-}$ mouse (*top*) and an infected Rag-$1^{-/-}$ recipient of $CD4^+CD25^-$ T cells isolated from an infected donor (*bottom*). The adoptively transferred cells migrate to the site of infection and populate the lamina propria in a pattern similar to that observed in wild-type mice with severe pathology. *E*, Histopathological analysis of all mice of the adoptive transfer study shown in *A*, with regard to the four parameters: chronic inflammation, atrophy, intestinal metaplasia, and hyperplasia. The study included five infected control mice, four uninfected recipients of $CD4^+CD25^-$ T cells, and five infected recipients of $CD4^+CD25^-$ T cells; two uninfected control mice that did not receive cells are not shown. Their histopathology scores were all 0. *F*, Histopathological analysis of all mice of the adoptive transfer study shown in *B*, with regard to the four parameters: chronic inflammation, atrophy, intestinal metaplasia, and hyperplasia. The study included four infected control mice, three infected recipients of $CD4^+CD25^-$ T cells from an uninfected donor, and six infected recipients of $CD4^+CD25^-$ T cells from an infected donor; again, two uninfected control mice that did not receive cells are not shown, because their histopathology scores were all 0. The two studies shown in *E* and *F* are representative of several similarly designed studies that were conducted in either the Rag-$1^{-/-}$ or TCR-$\beta^{-/-}$ backgrounds. The statistical significance of all comparisons as calculated with Student's *t* test is indicated by *p* values; n.s., not significant. Thin bars in *E* and *F* indicate the means. The color code is the same in *A* and *E* and in *B* and *F*.

bars) and the rapid appearance of premalignant lesions in the stomach (Fig. 7, *C*, *E*, and *F*, inf. + $CD4^+_{inf}$). Both elevated gastric IFN-γ and pathology was strictly dependent on *Helicobacter* infection of the recipient, as cells from an infected donor had no effect in an uninfected recipient (Fig. 7, *A*, *C*, and *E*, uninf. + $CD4^+_{inf}$). Furthermore, cells isolated from an uninfected donor failed to clear *Helicobacter* infection in the recipient (Fig. 7*B*) and were also ineffective in inducing gastric pathology (Fig. 7, *C* and *F*, inf. + $CD4^+_{uninf}$) or IFN-γ responses (Fig. 7*B*). In conclusion, $CD4^+CD25^-$ T cell-induced clearance and concomitant pathology depends on infection both on the part of the donor and on the part of the recipient, suggesting that the prior priming and correct homing of transferred $CD4^+CD25^-$ T cells is crucial for both phenotypes. Indeed, staining for the CD4 surface marker reveals massive infiltration of these cells into the (infected) gastric mucosa, where their patterns of lamina propria colonization are strikingly similar to those observed in wild-type mice with severe pathology (Figs. 7*D* and 2*C*). Interestingly, production of the murine functional IL-8 analog MIP-2 is independent of infection on the part of the donor (but not the recipient, Fig. 7, *A* and *B*), as adoptive transfer of cells from an uninfected donor can trigger MIP-2 expression at similar levels as those from an infected donor. In contrast to the strong effects that adoptive transfer of $CD4^+$ cells has on immunodeficient recipients, we have not observed any such effects upon transfer of purified $CD8^+$ T cell populations, even when much higher numbers of cells were transferred (up to 2×10^6 cells). This result suggests that $CD8^+$ T cells do not contribute to *Helicobacter* control or gastric pathology in a measurable way in this model.

IFN-γ production by $CD4^+CD25^-$ effector T cells is essential for efficient clearance of Helicobacter

The control of *Helicobacter* by $CD4^+CD25^-$ effector T cells in the adoptive transfer model is accompanied by the production of large quantities of local IFN-γ (Fig. 7, *A* and *B*). To assess the contribution of effector T cell-derived IFN-γ to bacterial clearance and concomitant immunopathology, we adoptively transferred wild-type or IFN-$\gamma^{-/-}$ $CD4^+CD25^-$ effector T cells from infected donors into immunodeficient *Helicobacter*-infected hosts. Whereas the recipients of wild-type cells efficiently cleared the infection, the recipients of IFN-$\gamma^{-/-}$ cells were still heavily colonized 4 wk later (Fig. 8*A* showing *flaB*- and *ureB*-specific PCRs and quantitative *flaB* PCR performed on gastric genomic DNA), suggesting that IFN-γ production by effector T cells is essential for clearance. Indeed, IFN-γ expression in gastric tissue of IFN-$\gamma^{-/-}$ cell recipients was minimal (Fig. 8*A, middle panel*), implying that $CD4^+CD25^-$ effector T cells are the main producers of the cytokine in this setting. In contrast, the neutrophil chemoattractant MIP-2 was detectable in moderate amounts also in the gastric mucosa of IFN-$\gamma^{-/-}$ cell recipients, suggesting that it is produced (or induced) also by IFN-$\gamma^{-/-}$ T cells (Fig. 8*A*). This observation led us to quantify the levels of neutrophil infiltration in all groups of mice by an assay measuring the activity of myeloperoxidase, an enzyme produced in large quantities by neutrophils and, to a lesser extent, monocytes and macrophages. Gastric neutrophil infiltration proved to be clearly dependent on adoptively transferred $CD4^+CD25^-$ effector T cells, as there was only minor infiltration due to infection alone (Fig. 8*B*). However, neutrophil infiltration was virtually identical in the two groups that had received wild-type or IFN-$\gamma^{-/-}$ T cells, suggesting that IFN-γ does not affect neutrophil recruitment, but may rather act by modulating neutrophil phagocytic or microbicidal activity, survival, or other characteristics.

We further assessed the role of effector T cell-derived IFN-γ in *Helicobacter*-associated gastric pathology as a function of

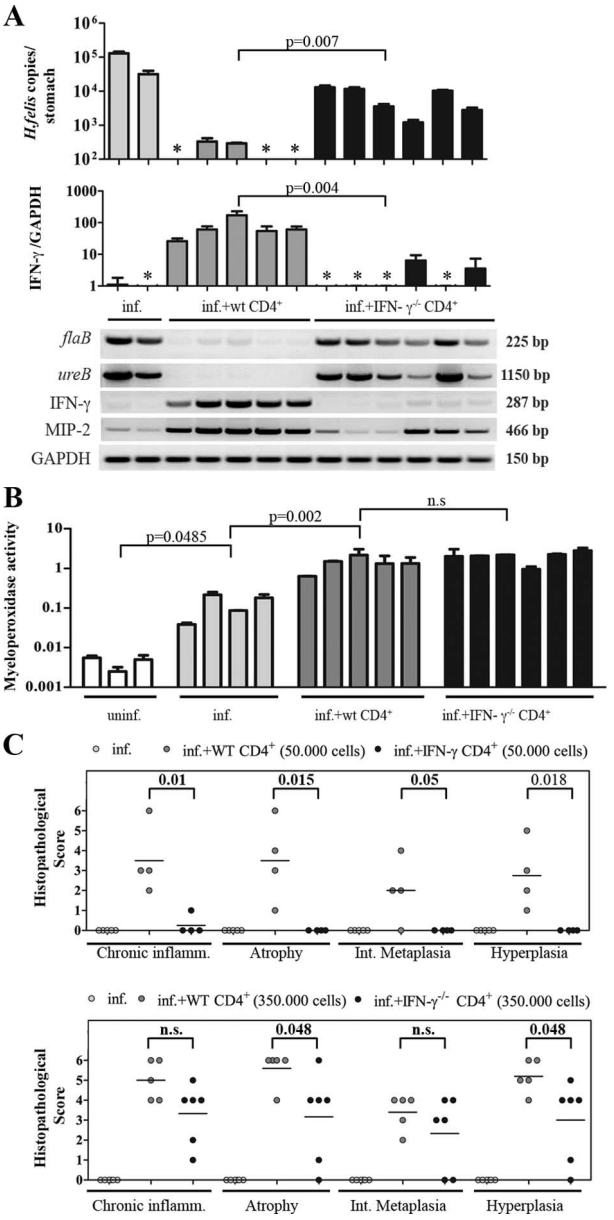


FIGURE 8. IFN-γ produced by adoptively transferred $CD4^+CD25^-$ T cells is essential for efficient clearance of *Helicobacter*. Six-week-old immunodeficient TCR-$\beta^{-/-}$ mice received 350,000 immunomagnetically purified $CD4^+CD25^-$ T cells from infected wild-type (WT) or IFN-$\gamma^{-/-}$ donors as indicated. The recipients were infected (inf.) with *H. felis* 1 day later and were sacrificed 1 mo later. Infected TCR-$\beta^{-/-}$ mice that had not received cells served as controls. All tissues were analyzed as described in the legend to Fig. 7. *A*, Colonization and transcript levels as determined by conventional and real-time PCR or RT-PCR. Only mice that have received wild-type $CD4^+CD25^-$ T cells are able to clear the infection and launch a strong IFN-γ response. Asterisks indicate values that were below the detection limit. Values of *p* were calculated the by Mann-Whitney *U* test. *B*, Myeloperoxidase assay reflecting the influx of neutrophils and, to a lesser extent, monocytes and macrophages into the gastric mucosa. Duplicate readings were performed for extracts of every mouse and averages as well as SDs are plotted. A representative myeloperoxidase assay of three is shown. The animals plotted are the same as in *A*; three uninfected mice and two additional infected mice are also included. Values of *p* were calculated using Student's *t* test. *C*, Histopathological analysis of the study shown in *A* and *B* (*lower panel*, 350,000 cells transferred) as well as an independent study using fewer cells (*upper panel*, 50,000 cells transferred). Group sizes ranged from four to seven mice. Values of *p* were calculated using Student's *t* test. Thin bars in *C* indicate the means. The color code is the same in *A–C*. Two representative experiments of three are shown. n.s., Not significant.

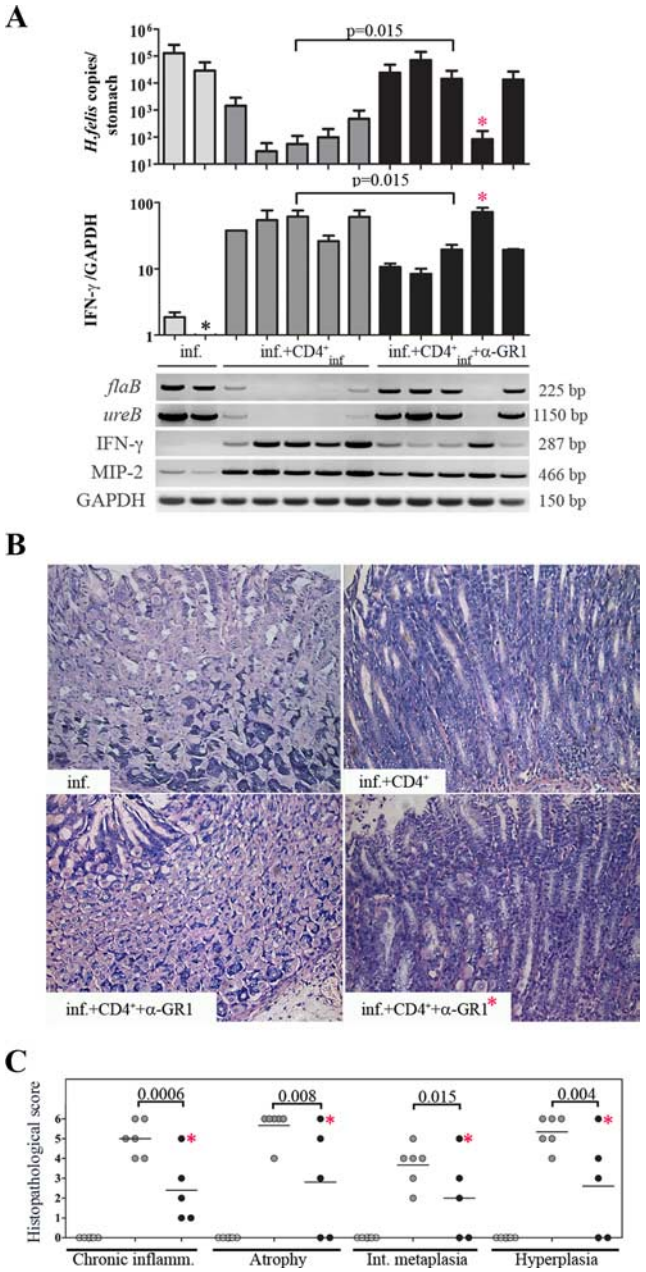


FIGURE 9. Neutrophils are essential for *Helicobacter* clearance in the adoptive transfer model. TCR-$\beta^{-/-}$ mice received 350,000 $CD4^+CD25^-$ T cells and were infected with *H. felis*. Neutrophil depletion was accomplished by three times weekly i.p. injections of 250 μg of GR-1-specific Ab (clone RB6-8C5) for the entire 4-wk duration of the experiment. All but one of the treated mice was devoid of neutrophils in the stomach as well as the spleen as assessed by immunohistochemistry using a GR-1-directed Ab (data not shown) upon termination of the experiment. The exceptional mouse in which the treatment failed is marked by a red asterisk. *A*, *flaB*- and *ureB*-specific (quantitative) PCRs were performed on genomic whole stomach DNA and IFN-γ, MIP-2, and GAPDH transcript levels were assessed by conventional and real-time RT-PCR on mucosal RNA as in Figs. 7 and 8. Anti-GR-1-treated mice failed to clear the infection and to launch a strong IFN-γ response. The *p* values were calculated by the Mann-Whitney *U* test. A gray asterisk indicates values below the detection limit. *B*, Representative sections are shown of TCR-$\beta^{-/-}$ mice that have been infected only (inf.) or have received $CD4^+CD25^-$ T cells, but no Ab treatment (inf. + $CD4^+$) or both the cells and the Ab treatment (inf. + $CD4^+$ + anti-GR-1). A section from the mouse in which the treatment failed is shown as well (marked by a red asterisk). *C*, Histopathology scores for all mice of the study. Group sizes were five mice each. The *p* values are calculated by Student's *t* test, excluding the mouse in which the treatment failed. Thin bars in *C* indicate the means.

the number of transferred cells. In an experiment in which we adoptively transferred only 50,000 cells into immunodeficient mice, we saw a significant difference in all four histopathological parameters when comparing recipients of wild-type vs IFN-$\gamma^{-/-}$ cells (Fig. 8*C, top panel*). In an independent transfer experiment with 350,000 cells, the differences were significant for atrophy and hyperplasia scores, but less clear for metaplasia (Fig. 8*C, bottom panel*). This result suggests that induction of gastric pathology, especially manifesting as atrophy accompanied by compensatory hyperplasia, largely depends on IFN-γ. The induction of metaplasia, in contrast, seems to be linked less clearly with IFN-γ, a finding that is in line with the less pronounced expression of this cytokine in predominantly metaplastic compared with hyperplastic tissues (Fig. 4, *B* and *C*) and the less compelling statistical link between IFN-γ expression and metaplasia compared with hyperplasia scores in wild-type mice (Fig. 3*D*).

Neutrophil granulocytes are essential for clearance of Helicobacter

Neither *H. felis* nor *H. pylori* are cleared spontaneously from experimentally infected mice; therefore, nonvaccine-induced clearance models are not readily available. Our model of clearance/reduction of *H. felis* upon adoptive transfer of $CD4^+CD25^-$ T cells provides a system in which effector mechanisms involved in efficient *Helicobacter* clearance can be studied. Because neutrophils have been implied in phagocytic removal of other mucosal pathogens, we depleted them for the duration of the experiment by treating the mice three times weekly with doses of 250 μg of an Ab targeting the neutrophil-specific surface marker GR-1. Depletion of neutrophils prevented clearance in all but one of five mice (Fig. 9*A*; the exceptional mouse later turned out not to have responded to the treatment, as neutrophil numbers were normal in the stomach and spleen) compared with a control group in which all five mice had cleared the infection. Interestingly, the gastric IFN-γ production indicative of T cell infiltration was also blocked in all four mice that had failed to clear due to successful Ab treatment (Fig. 9*A*), suggesting that neutrophils contribute to clearance of *Helicobacter* at least in part by recruiting more effector cells to the stomach. This reduced gastric infiltration of T cells is reflected also in a significant protection from preneoplasia (Fig. 9, *B* and *C*). To rule out that the GR-1 Ab acts directly on activated T cells and depletes them from the gastric mucosa, we monitored gastric T cell levels before and after treatment in immunocompetent mice with preexisting lesions. In this scenario, we did not observe unwanted effects of the Ab treatment on T cells, suggesting that the Ab affects neutrophils only (data not shown). In conclusion, neutrophils play an important role during clearance of *Helicobacter* by facilitating T lymphocyte recruitment and possibly also by phagocytic removal of luminal bacteria.

Discussion

In this study, we demonstrate a crucial role of IFN-γ for the control of *Helicobacter* infection and for induction of preneoplastic changes of the infected gastric mucosa. This dual role is evident in an experimental murine host that is capable of reducing the bacterial burden upon the onset of an adaptive immune response (but not capable of complete spontaneous clearance) and that therefore is also susceptible to ensuing immunopathology. We show that the extent of the IFN-γ response differs among genetically virtually identical animals (littermates) and that it is not only predictive of *Helicobacter* control and resulting gastric pathology, but is also causally associated with both. In addition to contributing to the

spontaneous reduction in bacterial burden in experimentally infected mice, a strong IFN-γ response additionally is characteristic of and required for protection induced by an *H. pylori* vaccine. Our data confirm and extend several early studies that have proposed a role for T_H1 cells (43, 44), and IFN-γ in particular, in the control of *H. pylori* infection upon vaccination (45) or primary infection (46). Several previous studies also noted reduced gastritis in infected IFN-$\gamma^{-/-}$ compared with wild-type mice (38, 44, 45), but did not assess preneoplastic epithelial pathology because it does not readily form after infection with *H. pylori* (as opposed to the more virulent *H. felis*). In conflict with our data and those of Akhiani et al. (45), two previous studies have not found a conclusive role for IFN-γ in vaccine-induced protection (38, 47), despite elucidating the contribution of IL-12, another T_H1 signature cytokine, to *H. pylori* clearance (47). Experimental differences between the four studies that have assessed the role of IFN-γ in protection so far include the challenge strain used, the time-to-sacrifice after challenge, the overall level of protection achieved by vaccination, and, perhaps most importantly, the gender of the vaccinated mice. We have observed clear differences in vaccine-induced protection in female and male wild-type mice (females are typically significantly better protected), as well as in spontaneous clearance models (i.e., IL-$10^{-/-}$ mice; A. Sayi and A. Müller, unpublished observation). Although the significance of this gender difference remains unclear, we suspect that it might provide an explanation for the conflicting results of the four studies. We should also stress that, although the differences in protection between wild-type and IFN-$\gamma^{-/-}$ animals are clearly significant in our hands ($p = 0.0014$), the defect in clearance of the IFN-$\gamma^{-/-}$ strain is much less pronounced than the defect of MHCII$^{-/-}$ or CD4$^{-/-}$ strains lacking CD4$^+$ T cells entirely (which are colonized as well as unvaccinated controls; I. Hitzler and A. Müller, unpublished data).

In the vaccine model, the successful control of *Helicobacter* colonization upon immunization is accompanied by considerable gastritis and mild atrophy at the antrum/corpus junction (Fig. 6). This so-called "postimmunization" gastritis is of concern in *Helicobacter* vaccine development (41), as none of the published vaccination approaches achieves sterile immunity. Indeed, we did not observe a regression of vaccine-induced pathology even 7 wk after challenge (i.e., 5 wk after a significant reduction in bacterial loads; Fig. 6), confirming published observations (41). We have shown previously in a long-term vaccine study that *H. felis*-induced MALT lymphoma formation in BALB/c mice is prevented by vaccination (48). Similarly, Sutton et al. (41) have demonstrated that at least the mild pathology triggered by *H. pylori* SS1 infection in C57BL/6 mice is not exacerbated upon vaccination in the long run. Nevertheless, the issue of whether vaccination and challenge with more immunogenic strains (better mimicking the human-pathogen interaction) will induce unacceptable preneoplastic pathology in susceptible (e.g., C57BL/6) hosts because the bacteria are reduced but not eliminated remains unsolved and certainly requires future attention if *Helicobacter* vaccination is to become a reality. In any case, our data argue that the distinction between "pathogenic T cells" and "protective T cells" may not be correct and that the protective (vaccine-induced) vs natural responses to the infection are quantitatively rather than qualitatively different (as discussed in Ref. 49).

Multiple cell types are capable of producing IFN-γ during both the innate and adaptive phases of the immune response. By depletion of NK and NKT cells from experimentally infected mice, we have ruled out a crucial contribution of these two cell types in the control of *Helicobacter* infection. Rather, we observed that CD4$^+$CD25$^-$ effector T cells produce large amounts of IFN-γ when adoptively transferred into immunodeficient recipients (Rag-$1^{-/-}$ or TCR-$\beta^{-/-}$). In these adoptive transfer models, the recipients clear or reduce the infection and develop severe premalignant pathology, as was also documented recently by Lee et al. (50); IFN-γ is essential for both effects since cells from an IFN-$\gamma^{-/-}$ background fail to eliminate the infection and are less potent than wild-type cells in initiating gastric pathology (Fig. 8). IFN-γ production and CD4$^+$CD25$^-$ effector cell-mediated pathology (as well as clearance) depend on *Helicobacter* infection on the part of the donor and on the part of the recipient (Fig. 7). In addition to NK-, NKT- and T_H1-polarized CD4$^+$ cells, CD8$^+$ T cells are known to be important producers of IFN-γ during the adaptive phase of an immune response and have been implicated previously in *Helicobacter*-induced gastritis (51). We thus tested their possible contribution to *Helicobacter* clearance and associated immunopathology in our adoptive transfer model. However, we found no evidence for gastric IFN-γ production, or for a reduction of bacterial burden, or for gastric pathology in CD8$^+$ T cell recipients, thereby ruling out an important effector function of CD8$^+$ T cells at least in this model.

IFN-γ is best known for its effects on macrophages and dendritic cells (DC), which are induced to present Ag in the context of MHC class II upon exposure (39). Depletion of macrophages with liposome-encapsulated chlodronate (a bisphosphonate toxin; data not shown) did not reduce vaccine efficacy, suggesting that macrophages are dispensable for the effector phase of *Helicobacter* clearance. The presence of IFN-γ during the TLR-dependent activation of DC has been shown to promote the production of IL-12, a cytokine which drives further T_H1 differentiation and amplifies the production of IFN-γ, thereby creating a positive feedback loop. According to two recent reports, DC residing in Peyer's patches of the small intestine prime a *Helicobacter*-specific T_H1-polarized immune response upon exposure to the coccoid form of *H. pylori* and direct migration of effector T cells to the gastric mucosa (52, 53). However, the role of DC in *Helicobacter* clearance upon vaccination remains poorly understood, mostly due to the lack of appropriate tools.

Interestingly, multiple lineages of gastric epithelial cells respond to IFN-γ exposure. Kang et al. (54) found that the human gastric cell line NCI-N87 acquired mucous neck cell traits upon treatment with IFN-γ such as secretion of mucus and expression of the mucin MUC6, TFF2, and pepsinogen II. Infusion of IFN-γ into wild-type mice was further shown to induce expansion of the mucous neck cell compartment in vivo, probably by triggering the deregulated proliferation of this cell type (54). In a study investigating a rare progenitor cell type of the antral gland by lineage tracing, Qiao et al. (55) observed that these cells have multilineage potential (i.e., they give rise to all lineages of the antral gland) and that they multiply in response to IFN-γ. In our own previous study investigating the response of the three terminally differentiated gastric epithelial lineages to *Helicobacter* infection in vivo using a laser capture approach, we found that the mucus-producing pit cell responds strongly to infection (56) by up-regulating numerous known target genes of the IFN-γ signaling pathway. We extended these findings in the current study by treating an immortalized gastric murine epithelial cell line with IFN-γ. Virtually all of the most strongly IFN-γ-induced genes in this line were coregulated with IFN-γ in preneoplastic gastric lesions, suggesting their induction by IFN-γ also in vivo. It is interesting to note in this context that predominantly hyperplastic, but not metaplastic, mucosa was characterized by high IFN-γ production (Fig. 4); this finding possibly suggests that only the former cell type expands as a result of IFN-γ exposure, whereas metaplasia may arise by an alternative mechanism. Additional evidence also points in this direction: metaplasia was the only one of the four histopathological

parameters evaluated that did not show a significant association with IFN-γ expression in a large group of wild-type mice with various degrees of pathology (Fig. 3*D*). It was also the only lesion that appeared in immunodeficient recipients of IFN-$\gamma^{-/-}$ $CD4^+$ effector cells (Fig. 8*C*). Concluding these findings, it is tempting to speculate that IFN-γ produced in the setting of chronic inflammation in the stomach acts directly on one or more epithelial lineages to induce their deregulated proliferation and transformation to premalignant, hyperplastic phenotypes. Whether epithelial cells are also involved in clearance of the bacteria is currently unknown.

Indeed, the downstream effector mechanisms leading to elimination of the bacteria from the infected stomach are not well understood, neither in spontaneous nor vaccine-induced clearance models. Whereas Abs seem to be dispensable (57), neutrophil granulocytes (58, 59) and mast cells (60) have been implied in *Helicobacter* clearance. Neutrophils in particular were found to be important in a spontaneous clearance model of *H. felis* infection in IL-$10^{-/-}$ mice (58), in a vaccine-induced clearance model (59), and in our $CD4^+$ T cell-mediated clearance model in immunodeficient mice (Fig. 9), suggesting a common downstream effector mechanism involving this cell type.

The CXC chemokine IL-8 (CXCL-8) and its functional murine counterpart MIP-2 are well known to induce neutrophil attraction, activation, and transendothelial migration. IL-8 is strongly induced in human gastric cancer cells such as the commonly used AGS cell line upon coculture with *H. pylori* (61–63), and this signal is generally assumed to initiate the acute (neutrophil-dominated) inflammation in the early stages of *Helicobacter* infection. Similarly, we observed that *H. pylori* infection triggers the production of MIP-2 in primary as well as immortalized murine gastric cells in vitro (I. Arnold and A. Müller, unpublished data). In vivo, IL-8 has been shown to be strongly induced in the infected gastric human mucosa and its levels are correlated positively with pathology (64). In accordance with these findings, we measured high levels of MIP-2 transcript in infected mice and we detected the presence of neutrophils in the gastric mucosa histologically and by ELISA (data not shown for wild-type mice). Neutrophils are sometimes found luminally and in close proximity to *Helicobacter* organisms (data not shown). Interestingly however, we observed only minor MIP-2 induction (Figs. 7–9) and neutrophil infiltration in mice lacking either T cells or T and B cells. Adoptive transfer of $CD4^+$ effector populations restores MIP-2 signals (Figs. 7–9) and neutrophil infiltration (Fig. 8) in these strains. Whether MIP-2 is produced by the T effector cells themselves in this scenario or its increased production by epithelial cells is triggered by lymphocyte-derived signals is currently not known. Either way, efficient gastric MIP-2 production and neutrophil recruitment depends on $CD4^+$ T cell infiltration in conjunction with infection (Fig. 7), but is independent of concomitant IFN-γ production (Fig. 8). In conclusion, our data are consistent with the following scenario: the initial contact of bacteria with gastric epithelial cells triggers moderate production of MIP-2. This instigates the modest infiltration and activation of neutrophils, which is itself a prerequisite for the subsequent recruitment of *Helicobacter*-specific T cells to the site of infection. These cells then generate the strong MIP-2 signals that are required to attract more neutrophils, which in turn accomplish clearance or reduction of the infection. IFN-γ secretion by $CD4^+$ T_H1 cells is crucial for clearance. Although being dispensable for the recruitment of neutrophils in our as well as other model systems (65), IFN-γ is known to induce neutrophil phagocytosis (66), microbicidal activity (67), and survival (68). We propose that $CD4^+$ T cell-derived IFN-γ must act locally on neutrophils to activate their anti-*Helicobacter* properties. In addition (or alternatively) to IFN-γ, another proinflammatory cytokine, IL-17, has recently been assigned an important function in the mucosal response to *Helicobacter* infection, in particular in neutrophil recruitment. IL-17 levels are increased in the human-infected mucosa (69) as well as in vaccinated mice (59), and this seems to initiate acute inflammation in the mouse model (70). However, a conclusive functional role for IL-17 in *Helicobacter* control has not yet been found; on the contrary, IL-$17^{-/-}$ mice seem to be less colonized than wild-type mice (70). Data on vaccine studies in IL-$17^{-/-}$ mice are not yet available, but will hopefully clarify soon whether IL-17 is important in vaccine-induced protection.

Being the signature cytokine of T_H1-biased Th responses, IFN-γ is efficiently down-regulated by T_H2 cytokines such as IL-4. Indeed, the BALB/c strain of mice that is known to predominantly mount T_H2-biased responses to infectious agents is completely protected from developing the preneoplastic lesions typically observed in the C57BL/6 background. Experimental polarization of systemic responses to *Helicobacter* by concurrent helminth infection was further shown to alleviate gastric premalignant pathology (71), lending considerable support to this model. In fact, an epidemiological study from Colombia looking at T_H2-polarized responses to *Helicobacter* and concurrent helminth infections in children suggested that populations with low gastric cancer risk had significantly higher rates of helminths than high-risk populations (72). Apart from environmental factors affecting T_H1/T_H2 balance, disease outcome upon chronic infection may be influenced by polymorphisms affecting IFN-γ signaling, for instance in the IFN-γ receptor chain 1 (*IFNGR*) or the *IFNG* gene itself (25, 73–75). Overall, both animal experimental and epidemiological evidence seems to increasingly imply the (T cell-driven) immune response to the infection as a driving force behind gastric preneoplasia. Our finding that Rag-$1^{-/-}$ and TCR-$\beta^{-/-}$ are completely protected from premalignant pathology is in line with this assumption. Although there is obviously a clear contribution of *Helicobacter* virulence factors (such as the type IV secretion substrate CagA) to gastric cancer risk (76), one may speculate that such factors act as immune modulators (e.g., by disrupting the epithelial barrier and thereby exacerbating inflammation (77)) rather than as true bacterial oncoproteins. Finally, additional, as yet unknown, factors seem to affect the type of immune response launched against the infection, as illustrated by the heterogeneity of responses observed in mice of the same gender and same age, infected for the same time with the same strain (Figs. 1 and 3). Although superinfections with other bacteria could be a contributing factor (although this may not be very likely for mice inhabiting the same cage for many months), we favor an explanation factoring in the "stress" component. We speculate that in groups of mice housed together (especially males, which were used mostly in our studies), the highest ranking male(s) might be the least stressed and, as a result, is least susceptible to *Helicobacter*-induced gastritis and pathology.

In conclusion, our results confirm and extend experimental and epidemiological data suggesting that IFN-γ plays an important role in the control of *Helicobacter* infection. At the same time, $CD4^+$ T cell-derived IFN-γ provides the key stimulus for development of gastric premalignant lesions that can progress to gastric cancer.

Acknowledgments

We thank Isabella Toller for help with the myeloperoxidase assay and comments on this manuscript, and Joe Jiricny and all members of the Institute for helpful discussions. We are grateful to the Functional Genomics Center Zurich for access to equipment and to Andrea Patrignani for assistance with microarray experiments. Trevor Lawley, Amita Kawale, and Pauline Chu are thanked for help with animal experimentation and

histology. A.M. wishes to thank Stanley Falkow for continued support and helpful discussions.

Disclosures

The authors have no financial conflict of interest.

References

1. Parsonnet, J., G. D. Friedman, D. P. Vandersteen, Y. Chang, J. H. Vogelman, N. Orentreich, and R. K. Sibley. 1991. *Helicobacter pylori* infection and the risk of gastric carcinoma. *N. Engl. J. Med.* 325: 1127–1131.
2. Parsonnet, J., S. Hansen, L. Rodriguez, A. B. Gelb, R. A. Warnke, E. Jellum, N. Orentreich, J. H. Vogelman, and G. D. Friedman. 1994. *Helicobacter pylori* infection and gastric lymphoma. *N. Engl. J. Med.* 330: 1267–1271.
3. Parsonnet, J., and P. G. Isaacson. 2004. Bacterial infection and MALT lymphoma. *N. Engl. J. Med.* 350: 213–215.
4. Danesh, J. 1999. Helicobacter pylori infection and gastric cancer: systematic review of the epidemiological studies. *Aliment Pharmacol. Ther.* 13: 851–856.
5. Marshall, B. J., and J. R. Warren. 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1: 1311–1315.
6. Pritchard, D. M., and J. E. Crabtree. 2006. *Helicobacter pylori* and gastric cancer. *Curr. Opin. Gastroenterol.* 22: 620–625.
7. Parkin, D. M., P. Pisani, and J. Ferlay. 1999. Estimates of the worldwide incidence of 25 major cancers in 1990. *Int. J. Cancer* 80: 827–841.
8. Murray, C. J., and A. D. Lopez. 1997. Alternative projections of mortality and disability by cause 1990–2020: Global Burden of Disease Study. *Lancet* 349: 1498–1504.
9. Lauren, P. 1965. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma: an attempt at a histo-clinical classification. *Acta Pathol. Microbiol. Scand.* 64: 31–49.
10. Grabiec, J., and D. A. Owen. 1985. Carcinoma of the stomach in young persons. *Cancer* 56: 388–396.
11. Chen, X., S. Y. Leung, S. T. Yuen, K. M. Chu, J. Ji, R. Li, A. S. Chan, S. Law, O. G. Troyanskaya, J. Wong, S. So, D. Botstein, and P. O. Brown. 2003. Variation in gene expression patterns in human gastric cancers. *Mol. Biol. Cell* 14: 3208–3215.
12. Correa, P. 1988. A human model of gastric carcinogenesis. *Cancer Res.* 48: 3554–3560.
13. Correa, P. 1995. *Helicobacter pylori* and gastric carcinogenesis. *Am. J. Surg. Pathol.* 19(Suppl. 1): S37–S43.
14. Correa, P., W. Haenszel, C. Cuello, S. Tannenbaum, and M. Archer. 1975. A model for gastric cancer epidemiology. *Lancet* 2: 58–60.
15. Baldwin, D. N., B. Shepherd, P. Kraemer, M. K. Hall, L. K. Sycuro, D. M. Pinto-Santini, and N. R. Salama. 2007. Identification of *Helicobacter pylori* genes that contribute to stomach colonization. *Infect. Immun.* 75: 1005–1016.
16. Sakagami, T., M. Dixon, J. O'Rourke, R. Howlett, F. Alderuccio, J. Vella, T. Shimoyama, and A. Lee. 1996. Atrophic gastric changes in both *Helicobacter felis* and *Helicobacter pylori* infected mice are host dependent and separate from antral gastritis. *Gut* 39: 639–648.
17. Fox, J. G., B. J. Sheppard, C. A. Dangler, M. T. Whary, M. Ihrig, and T. C. Wang. 2002. Germ-line p53-targeted disruption inhibits *Helicobacter*-induced premalignant lesions and invasive gastric carcinoma through down-regulation of Th1 proinflammatory responses. *Cancer Res.* 62: 696–702.
18. Enno, A., J. L. O'Rourke, C. R. Howlett, A. Jack, M. F. Dixon, and A. Lee. 1995. MALToma-like lesions in the murine gastric mucosa after long-term infection with *Helicobacter felis*: a mouse model of *Helicobacter pylori*-induced gastric lymphoma. *Am. J. Pathol.* 147: 217–222.
19. Enno, A., J. O'Rourke, S. Braye, R. Howlett, and A. Lee. 1998. Antigen-dependent progression of mucosa-associated lymphoid tissue (MALT)-type lymphoma in the stomach: effects of antimicrobial therapy on gastric MALT lymphoma in mice. *Am. J. Pathol.* 152: 1625–1632.
20. Mueller, A., J. O'Rourke, J. Grimm, K. Guillemin, M. F. Dixon, A. Lee, and S. Falkow. 2003. Distinct gene expression profiles characterize the histopathological stages of disease in *Helicobacter*-induced mucosa-associated lymphoid tissue lymphoma. *Proc. Natl. Acad. Sci. USA* 100: 1292–1297.
21. Mueller, A., J. O'Rourke, P. Chu, A. Chu, M. F. Dixon, D. M. Bouley, A. Lee, and S. Falkow. 2005. The role of antigenic drive and tumor-infiltrating accessory cells in the pathogenesis of *Helicobacter*-induced mucosa-associated lymphoid tissue lymphoma. *Am. J. Pathol.* 167: 797–812.
22. Fox, J. G., A. B. Rogers, M. Ihrig, N. S. Taylor, M. T. Whary, G. Dockray, A. Varro, and T. C. Wang. 2003. *Helicobacter pylori*-associated gastric cancer in INS-GAS mice is gender specific. *Cancer Res.* 63: 942–950.
23. Fox, J. G., T. C. Wang, A. B. Rogers, T. Poutahidis, Z. Ge, N. Taylor, C. A. Dangler, D. A. Israel, U. Krishna, K. Gaus, and R. M. Peek, Jr. 2003. Host and microbial constituents influence *Helicobacter pylori*-induced cancer in a murine model of hypergastrinemia. *Gastroenterology* 124: 1879–1890.
24. Vilaichone, R. K., V. Mahachai, S. Tumwasorn, J. Y. Wu, D. Y. Graham, and Y. Yamaoka. 2005. Gastric mucosal cytokine levels in relation to host interleukin-1 polymorphisms and *Helicobacter pylori* cagA genotype. *Scand. J. Gastroenterol.* 40: 530–539.
25. Rad, R., A. Dossumbekova, B. Neu, R. Lang, S. Bauer, D. Saur, M. Gerhard, and C. Prinz. 2004. Cytokine gene polymorphisms influence mucosal cytokine expression, gastric inflammation, and host specific colonisation during *Helicobacter pylori* infection. *Gut* 53: 1082–1089.
26. Sakai, K., M. Kita, N. Sawai, S. Shiomi, Y. Sumida, K. Kanemasa, S. Mitsufuji, J. Imanishi, and Y. Yamaoka. 2008. Levels of interleukin-18 are markedly increased in *Helicobacter pylori*-infected gastric mucosa among patients with specific IL18 genotypes. *J. Infect. Dis.* 197: 1752–1761.
27. Bontems, P., F. Robert, A. Van Gossum, S. Cadranel, and F. Mascart. 2003. *Helicobacter pylori* modulation of gastric and duodenal mucosal T cell cytokine secretions in children compared with adults. *Helicobacter* 8: 216–226.
28. El-Omar, E. M., M. Carrington, W. H. Chow, K. E. McColl, J. H. Bream, H. A. Young, J. Herrera, J. Lissowska, C. C. Yuan, N. Rothman, et al. 2000. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature* 404: 398–402.
29. El-Omar, E. M., C. S. Rabkin, M. D. Gammon, T. L. Vaughan, H. A. Risch, J. B. Schoenberg, J. L. Stanford, S. T. Mayne, J. Goedert, W. J. Blot, et al. 2003. Increased risk of noncardia gastric cancer associated with proinflammatory cytokine gene polymorphisms. *Gastroenterology* 124: 1193–1201.
30. Lee, A., J. O'Rourke, M. C. De Ungria, B. Robertson, G. Daskalopoulos, and M. F. Dixon. 1997. A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. *Gastroenterology* 112: 1386–1397.
31. Fujikawa, A., D. Shirasaka, S. Yamamoto, H. Ota, K. Yahiro, M. Fukada, T. Shintani, A. Wada, N. Aoyama, T. Hirayama, et al. 2003. Mice deficient in protein tyrosine phosphatase receptor type Z are resistant to gastric ulcer induction by VacA of *Helicobacter pylori*. *Nat. Genet.* 33: 375–381.
32. Tucker, M. S., E. M. Eves, B. H. Wainer, and M. R. Rosner. 1993. Activation of mitogen-activated protein kinase by epidermal growth factor in hippocampal neurons and neuronal cell lines. *J. Neurochem.* 61: 1376–1387.
33. Stoicov, C., M. Whary, A. B. Rogers, F. S. Lee, K. Klucevsek, H. Li, X. Cai, R. Saffari, Z. Ge, I. A. Khan, et al. 2004. Coinfection modulates inflammatory responses and clinical outcome of *Helicobacter felis* and *Toxoplasma gondii* infections. *J. Immunol.* 173: 3329–3336.
34. Dixon, M. F., R. M. Genta, J. H. Yardley, and P. Correa. 1996. Classification and grading of gastritis: The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am. J. Surg. Pathol.* 20: 1161–1181.
35. Chen, X. Y., R. W. van der Hulst, M. J. Bruno, A. van der Ende, S. D. Xiao, G. N. Tytgat, and F. J. Ten Kate. 1999. Interobserver variation in the histopathological scoring of *Helicobacter pylori* related gastritis. *J. Clin. Pathol.* 52: 612–615.
36. Mota, R. A., F. Sanchez-Bueno, L. Saenz, D. Hernandez-Espinosa, J. Jimeno, P. L. Tornel, A. Martinez-Torrano, P. Ramirez, P. Parrilla, and J. Yelamos. 2005. Inhibition of poly(ADP-ribose) polymerase attenuates the severity of acute pancreatitis and associated lung injury. *Lab. Invest.* 85: 1250–1262.
37. Torsten Hothorn, K. H., Mark A. van de Wiel, and Achim Zeileis. 2006. A Lego system for conditional inference. *Am. Statistician* 60: 257–263.
38. Sawai, N., M. Kita, T. Kodama, T. Tanahashi, Y. Yamaoka, Y. Tagawa, Y. Iwakura, and J. Imanishi. 1999. Role of γ interferon in *Helicobacter pylori*-induced gastric inflammatory responses in a mouse model. *Infect. Immun.* 67: 279–285.
39. Dalton, D. K., S. Pitts-Meek, S. Keshav, I. S. Figari, A. Bradley, and T. A. Stewart. 1993. Multiple defects of immune cell function in mice with disrupted interferon-γ genes. *Science* 259: 1739–1742.
40. Zeisberger, S. M., B. Odermatt, C. Marty, A. H. Zehnder-Fjallman, K. Ballmer-Hofer, and R. A. Schwendener. 2006. Clodronate-liposome-mediated depletion of tumour-associated macrophages: a new and highly effective antiangiogenic therapy approach. *Br. J. Cancer* 95: 272–281.
41. Sutton, P., S. J. Danon, M. Walker, L. J. Thompson, J. Wilson, T. Kosaka, and A. Lee. 2001. Post-immunisation gastritis and Helicobacter infection in the mouse: a long term study. *Gut* 49: 467–473.
42. Sporri, R., N. Joller, U. Albers, H. Hilbi, and A. Oxenius. 2006. MyD88-dependent IFN-γ production by NK cells is key for control of *Legionella pneumophila* infection. *J. Immunol.* 176: 6162–6171.
43. Mohammadi, M., S. Czinn, R. Redline, and J. Nedrud. 1996. *Helicobacter*-specific cell-mediated immune responses display a predominant Th1 phenotype and promote a delayed-type hypersensitivity response in the stomachs of mice. *J. Immunol.* 156: 4729–4738.
44. Smythies, L. E., K. B. Waites, J. R. Lindsey, P. R. Harris, P. Ghiara, and P. D. Smith. 2000. *Helicobacter pylori*-induced mucosal inflammation is Th1 mediated and exacerbated in IL-4, but not IFN-γ, gene-deficient mice. *J. Immunol.* 165: 1022–1029.
45. Akhiani, A. A., J. Pappo, Z. Kabok, K. Schon, W. Gao, L. E. Franzen, and N. Lycke. 2002. Protection against *Helicobacter pylori* infection following immunization is IL-12-dependent and mediated by Th1 cells. *J. Immunol.* 169: 6977–6984.
46. Kamradt, A. E., M. Greiner, P. Ghiara, and S. H. Kaufmann. 2000. *Helicobacter pylori* infection in wild-type and cytokine-deficient C57BL/6 and BALB/c mouse mutants. *Microbes Infect.* 2: 593–597.
47. Garhart, C. A., F. P. Heinzel, S. J. Czinn, and J. G. Nedrud. 2003. Vaccine-induced reduction of *Helicobacter pylori* colonization in mice is interleukin-12 dependent but γ interferon and inducible nitric oxide synthase independent. *Infect. Immun.* 71: 910–921.
48. Mueller, A., J. O'Rourke, P. Chu, C. C. Kim, P. Sutton, A. Lee, and S. Falkow. 2003. Protective immunity against *Helicobacter* is characterized by a unique transcriptional signature. *Proc. Natl. Acad. Sci. USA* 100: 12289–12294.
49. Blanchard, T. G., J. C. Eisenberg, and Y. Matsumoto. 2004. Clearance of *Helicobacter pylori* infection through immunization: the site of T cell activation contributes to vaccine efficacy. *Vaccine* 22: 888–897.
50. Lee, C. W., V. P. Rao, A. B. Rogers, Z. Ge, S. E. Erdman, M. T. Whary, and J. G. Fox. 2007. Wild-type and interleukin-10-deficient regulatory T cells reduce effector T-cell-mediated gastroduodenitis in $Rag2^{-/-}$ mice, but only wild-type regulatory T cells suppress *Helicobacter pylori* gastritis. *Infect. Immun.* 75: 2699–2707.

51. Tan, M. P., J. Pedersen, Y. Zhan, A. M. Lew, M. J. Pearse, O. L. Wijburg, and R. A. Strugnell. 2008. CD8+ T cells are associated with severe gastritis in *Helicobacter pylori*-infected mice in the absence of $CD4^+$ T cells. *Infect. Immun.* 76: 1289–1297.
52. Kiriya, K., N. Watanabe, A. Nishio, K. Okazaki, M. Kido, K. Saga, J. Tanaka, T. Akamatsu, S. Ohashi, M. Asada, et al. 2007. Essential role of Peyer's patches in the development of *Helicobacter*-induced gastritis. *Int. Immunol.* 19: 435–446.
53. Nagai, S., H. Mimuro, T. Yamada, Y. Baba, K. Moro, T. Nochi, H. Kiyono, T. Suzuki, C. Sasakawa, and S. Koyasu. 2007. Role of Peyer's patches in the induction of *Helicobacter pylori*-induced gastritis. *Proc. Natl. Acad. Sci. USA* 104: 8971–8976.
54. Kang, W., S. Rathinavelu, L. C. Samuelson, and J. L. Merchant. 2005. Interferon γ induction of gastric mucous neck cell hypertrophy. *Lab. Invest.* 85: 702–715.
55. Qiao, X. T., J. W. Ziel, W. McKimpson, B. B. Madison, A. Todisco, J. L. Merchant, L. C. Samuelson, and D. L. Gumucio. 2007. Prospective identification of a multilineage progenitor in murine stomach epithelium. *Gastroenterology* 133: 1989–1998.
56. Mueller, A., D. S. Merrell, J. Grimm, and S. Falkow. 2004. Profiling of microdissected gastric epithelial cells reveals a cell type-specific response to *Helicobacter pylori* infection. *Gastroenterology* 127: 1446–1462.
57. Ermak, T. H., P. J. Giannasca, R. Nichols, G. A. Myers, J. Nedrud, R. Weltzin, C. K. Lee, H. Kleanthous, and T. P. Monath. 1998. Immunization of mice with urease vaccine affords protection against *Helicobacter pylori* infection in the absence of antibodies and is mediated by MHC class II-restricted responses. *J. Exp. Med.* 188: 2277–2288.
58. Ismail, H. F., P. Fick, J. Zhang, R. G. Lynch, and D. J. Berg. 2003. Depletion of neutrophils in $IL\text{-}10^{-/-}$ mice delays clearance of gastric *Helicobacter* infection and decreases the Th1 immune response to *Helicobacter*. *J. Immunol.* 170: 3782–3789.
59. DeLyria, E. S., R. W. Redline, and T. G. Blanchard. 2009. Vaccination of mice against *H. pylori* induces a strong Th-17 response and immunity that is neutrophil dependent. *Gastroenterology* 136: 247–256.
60. Velin, D., D. Bachmann, H. Bouzourene, and P. Michetti. 2005. Mast cells are critical mediators of vaccine-induced *Helicobacter* clearance in the mouse model. *Gastroenterology* 129: 142–155.
61. Selbach, M., S. Moese, T. F. Meyer, and S. Backert. 2002. Functional analysis of the *Helicobacter pylori* cag pathogenicity island reveals both VirD4-CagA-dependent and VirD4-CagA-independent mechanisms. *Infect. Immun.* 70: 665–671.
62. Segal, E. D., C. Lange, A. Covacci, L. S. Tompkins, and S. Falkow. 1997. Induction of host signal transduction pathways by *Helicobacter pylori*. *Proc. Natl. Acad. Sci. USA* 94: 7595–7599.
63. Fischer, W., J. Puls, R. Buhrdorf, B. Gebert, S. Odenbreit, and R. Haas. 2001. Systematic mutagenesis of the *Helicobacter pylori* cag pathogenicity island: essential genes for CagA translocation in host cells and induction of interleukin-8. *Mol. Microbiol.* 42: 1337–1348.
64. Xuan, J., R. Deguchi, H. Yanagi, H. Ozawa, T. Urano, Y. Ogawa, R. Fukuda, S. Kojima, M. Nishina, H. Sudo, et al. 2005. Relationship between gastric mucosal IL-8 levels and histological gastritis in patients with *Helicobacter pylori* infection. *Tokai J. Exp. Clin. Med.* 30: 83–88.
65. Moreno, S. E., J. C. Alves-Filho, T. M. Alfaya, J. S. da Silva, S. H. Ferreira, and F. Y. Liew. 2006. IL-12, but not IL-18, is critical to neutrophil activation and resistance to polymicrobial sepsis induced by cecal ligation and puncture. *J. Immunol.* 177: 3218–3224.
66. Pearl-Yafe, M., I. Fabian, D. Halperin, E. Flatau, S. Werber, and I. Shalit. 2007. Interferon-γ and bacterial lipopolysaccharide act synergistically on human neutrophils enhancing interleukin-8, interleukin-1β, tumor necrosis factor-α, and interleukin-12 p70 secretion and phagocytosis via upregulation of Toll-like receptor 4. *Shock* 27: 226–231.
67. Rodrigues, D. R., L. A. Dias-Melicio, S. A. Calvi, M. T. Peracoli, and A. M. Soares. 2007. *Paracoccidioides brasiliensis* killing by IFN-γ, TNF-α and GM-CSF activated human neutrophils: role for oxygen metabolites. *Med. Mycol.* 45: 27–33.
68. Yoshimura, T., and M. Takahashi. 2007. IFN-γ-mediated survival enables human neutrophils to produce MCP-1/CCL2 in response to activation by TLR ligands. *J. Immunol.* 179: 1942–1949.
69. Caruso, R., D. Fina, O. A. Paoluzi, G. Del Vecchio Blanco, C. Stolfi, A. Rizzo, F. Caprioli, M. Sarra, F. Andrei, M. C. Fantini, et al. 2008. IL-23-mediated regulation of IL-17 production in *Helicobacter pylori*-infected gastric mucosa. *Eur. J. Immunol.* 38: 470–478.
70. Shiomi, S., A. Toriie, S. Imamura, H. Konishi, S. Mitsufuji, Y. Iwakura, Y. Yamaoka, H. Ota, T. Yamamoto, J. Imanishi, and M. Kita. 2008. IL-17 is involved in *Helicobacter pylori*-induced gastric inflammatory responses in a mouse model. *Helicobacter* 13: 518–524.
71. Fox, J. G., P. Beck, C. A. Dangler, M. T. Whary, T. C. Wang, H. N. Shi, and C. Nagler-Anderson. 2000. Concurrent enteric helminth infection modulates inflammation and gastric immune responses and reduces *Helicobacter*-induced gastric atrophy. *Nat. Med.* 6: 536–542.
72. Whary, M. T., N. Sundina, L. E. Bravo, P. Correa, F. Quinones, F. Caro, and J. G. Fox. 2005. Intestinal helminthiasis in Colombian children promotes a Th2 response to *Helicobacter pylori*: possible implications for gastric carcinogenesis. *Cancer Epidemiol. Biomarkers Prev.* 14: 1464–1469.
73. Thye, T., G. D. Burchard, M. Nilius, B. Muller-Myhsok, and R. D. Horstmann. 2003. Genomewide linkage analysis identifies polymorphism in the human interferon-gamma receptor affecting *Helicobacter pylori* infection. *Am. J. Hum. Genet.* 72: 448–453.
74. Zambon, C. F., D. Basso, F. Navaglia, C. Belluco, A. Falda, P. Fogar, E. Greco, N. Gallo, M. Rugge, F. Di Mario, and M. Plebani. 2005. Pro- and anti-inflammatory cytokines gene polymorphisms and *Helicobacter pylori* infection: interactions influence outcome. *Cytokine* 29: 141–152.
75. Canedo, P., G. Corso, F. Pereira, N. Lunet, G. Suriano, C. Figueiredo, C. Pedrazzani, H. Moreira, H. Barros, F. Carneiro, et al. 2008. The interferon γ receptor 1 (IFNGR1)-56C/T gene polymorphism is associated with increased risk of early gastric carcinoma. *Gut* 57: 1504–1508.
76. Huang, J. Q., G. F. Zheng, K. Sumanac, E. J. Irvine, and R. H. Hunt. 2003. Meta-analysis of the relationship between cagA seropositivity and gastric cancer. *Gastroenterology* 125: 1636–1644.
77. Amieva, M. R., R. Vogelmann, A. Covacci, L. S. Tompkins, W. J. Nelson, and S. Falkow. 2003. Disruption of the epithelial apical-junctional complex by *Helicobacter pylori* CagA. *Science* 300: 1430–1434.

3.2 Protective and pathogenic functions of T-cells are inseparable during the *Helicobacter*-host interaction

article published in Discovery Medicine, 2009

authors: Anne Müller, Ayca Sayi and Iris Hitzler

contributions: I contributed the data in the figure with help by AS, AM wrote the article.

Protective and Pathogenic Functions of T-cells Are Inseparable During the *Helicobacter*-host Interaction

ANNE MUELLER, AYCA SAYI, AND IRIS HITZLER

Abstract: **Chronic infection with the bacterial pathogen *Helicobacter pylori* is closely linked to the development of gastric cancer. Experimental infection of the laboratory mouse strain C57Bl6 mimics the initiation and progression of the disease in humans. Using this model, we have identified a dual role for $CD4^+$ IFN-γ-secreting T-cells in the control of *Helicobacter* infection as well as in the induction of preneoplastic gastric pathology. High gastric expression of IFN-γ was positively correlated with a low *Helicobacter* burden, and was essential for vaccine-induced protection; on the other hand, elevated levels of the cytokine also, either directly or indirectly, triggered the transformation of the normal gastric mucosa to atrophic, hyperplastic and metaplastic lesions. Based on similar patterns of gene expression changes induced by IFN-γ *in vivo* and in cultured gastric epithelial cells, we hypothesize that IFN-γ may act directly on epithelial cells to stimulate their hyperproliferation, and thus to predispose them to elevated mutation rates and an increased risk of malignant transformation.**

The bacterial pathogen *Helicobacter pylori* infects an estimated 50% of the world's population and has been associated with humans for tens of thousands of years, making it one of the most successful human bacterial pathogens of all time. Since its initial description by Marshall and Warren in 1984 (Marshall and Warren, 1984), *H. pylori* has been linked not only to chronic gastritis, which it causes, often asymptomatically, in all infected individuals, but also to the development of gastric ulcers and two distinct gastric malignancies, gastric cancer/adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (Parsonnet et al., 1991; 1994; 1997). Cancer does not arise until decades after the initial infection, and is believed to advance via a series of precursor lesions, all of which have independently been linked to *H. pylori* and to an elevated gastric cancer risk (Correa et al., 1975; 1988; 1995). Despite the fact that only a small fraction of infected individuals develop gastric cancer (most estimates are below 2%), and that infection rates have decreased substantially in most parts of the developed world in the second half of the 20th century, *Helicobacter* infection remains a large public health problem. The development of an *H. pylori* specific vaccine is widely viewed as a promising strategy of gastric cancer prevention.

Several small rodent models mimic the sequence of events leading up to gastric cancer in humans. Chronic infection of the Mongolian gerbil with *H. pylori* strains harboring the Cag pathogenicity island (Cag-PAI) causes ulcers as well as the precancerous lesions gastritis cystica profunda and focal dysplasia in a majority of animals within one year of initial infection (Wiedemann et al., 2009). In C57Bl6 mice, the closely related *Helicobacter*

***Anne Mueller**, Ph.D., **Ayca Sayi**, and **Iris Hitzler**, **M.S.**, are at the Institute of Molecular Cancer Research, University of Zurich, Zurich 8057, Switzerland.*

felis triggers early precursor lesions such as chronic atrophic gastritis, intestinal metaplasia, and pit cell hyperplasia within three months of infection (Sayi et al., 2009); these lesions can progress to dysplasia and *in situ* carcinoma after about one year (Fox et al., 2002). The C57Bl6 mouse model has been used extensively to dissect the events preceding epithelial transformation, and to understand how specific elements of the host's immune response contribute to the histopathological changes associated with an elevated gastric cancer risk. In a series of earlier studies, J. Fox and colleagues have reported a positive association of a high salt diet, hypergastrinemia and male gender on *Helicobacter*-induced gastric cancer and its precursor lesions (Fox et al., 2003a; 2003b). Fox et al. also proposed a role for Th1 biased immune responses, characterized by high levels of serum IgG2a and elevated local production of Th1 signature cytokines, in gastric cancer progression; in support of this hypothesis, experimental systemic Th2 polarization achieved by concomitant helminth infection attenuated the premalignant gastric pathology triggered by *Helicobacter* (Fox et al., 2000).

We have recently extended these findings in the C57Bl6 mouse model and reported a robust association between the local gastric production of the Th1 signature cytokine interferon-γ (IFN-γ) and the onset of preneoplasia, evident predominantly as atrophy of the specialized gastric epithelium and its replacement by hyperproliferating pit cell precursors (termed compensatory epithelial hyperplasia; Sayi et al., 2009). The study was based on our initial observation that genetically virtually identical individuals (littermates of the same gender) showed substantial heterogeneity in terms of gastric histopathology and *Helicobacter* colonization. Whereas a majority of mice managed to control the infection, and, as a result, developed moderate to severe gastric (immuno-) pathology as early as three months post infection, every group usually contained several animals that deviated from this pattern. These mice were heavily colonized but largely protected from gastric pathology. Further analysis of the gastric inflammatory cytokine profiles and lymphocyte infiltration patterns revealed that elevated IFN-γ mRNA and protein levels, as well as a diffuse infiltrate of lamina propria-invading $CD4^+$ T-cells, correlated significantly with symptoms of atrophy and hyperplasia, and were anti-correlated in all instances with *Helicobacter* colonization levels. The lesions appeared locally, affecting only the glands in the immediate vicinity of infiltrating T-cells. We therefore concluded that diffusely infiltrating, Th1 polarized T-cells contribute to the effective control of the infection, but are detrimental to the surrounding tissue. Further support for a direct role of IFN-γ both in the control of *Helicobacter* and in the induction of gastric epithelial changes came from the analysis of IFN-$\gamma^{-/-}$ mice. While these mice were colonized by an order of magnitude more heavily than wild type animals, they proved to be completely resistant to gastric pathology. Indeed, we were not able to find any evidence for a direct tissue-damaging effect of the bacteria in the absence of a T-cell mediated immune response, as TCR-$\beta^{-/-}$ and Rag-$1^{-/-}$ mice (which lack the TCRα/β positive subset and T- and B-cells, respectively) were also completely protected from gastric preneoplasia despite a heavy *Helicobacter* burden.

The evidence for a dual role of $CD4^+$ T-cells in *Helicobacter* control and the associated pathology led us to examine IFN-γ levels and gastric pathology under conditions of vaccine-induced *H. pylori* clearance. Using an experimental, orally administered *H. pylori* vaccine consisting of a whole cell sonicate adjuvanted by cholera toxin, we achieved a reduction of bacterial colonization by ~2.5 orders of magnitude upon challenge infection with the autologous strain. This vaccine-induced protection, which, importantly, failed to achieve sterilizing immunity, was accompanied by gastric IFN-γ levels at least 10 times higher than those measured upon infection of non-vaccinated control mice. IFN-$\gamma^{-/-}$ mice were significantly less protected than wild type mice. As might be predicted under these circumstances, the mild gastritis observed immediately upon challenge infection in wild type mice (2 weeks p.i.) progressed to moderate or severe atrophy accompanied by epithelial hyperplasia at later time points. Strikingly, vaccinated/challenged mice received significantly higher pathology scores than their infected but non-immunized counterparts at three months post challenge infection (**Figure 1**), implying that vaccination as a strategy of gastric cancer prevention will remain ineffective as long as sterilizing immunity is not achieved. No sterilizing vaccine regimens targeting *H. pylori* have been reported to date, despite substantial efforts made in the last decade at identifying novel *H. pylori* target

antigens (Nystrom and Svennerholm, 2007) and suitable adjuvants (Skene et al., 2008).

The reasons for the remarkable ability of *H. pylori* to persist in the human, as well as the rodent stomach for the host's lifetime, even in the face of a strong local inflammatory and adaptive immune response, remain incompletely understood. Whereas some investigators have advocated the existence of an intracellular niche (Amieva et al., 2002; Oh et al., 2005), either in terminally differentiated or in gastric tissue stem cells, others favor an explanation involving regulatory T-cells (Tregs; Zhang et al., 2008). Indeed, depletion of Tregs may improve vaccine efficacy (at least of suboptimal vaccination regimens achieving a reduction of less than one order of magnitude; Zhang et al., 2008). Furthermore, mice that have been depleted of Tregs (A. Sayi and A. Mueller, unpublished information) or that lack Treg effector molecules such

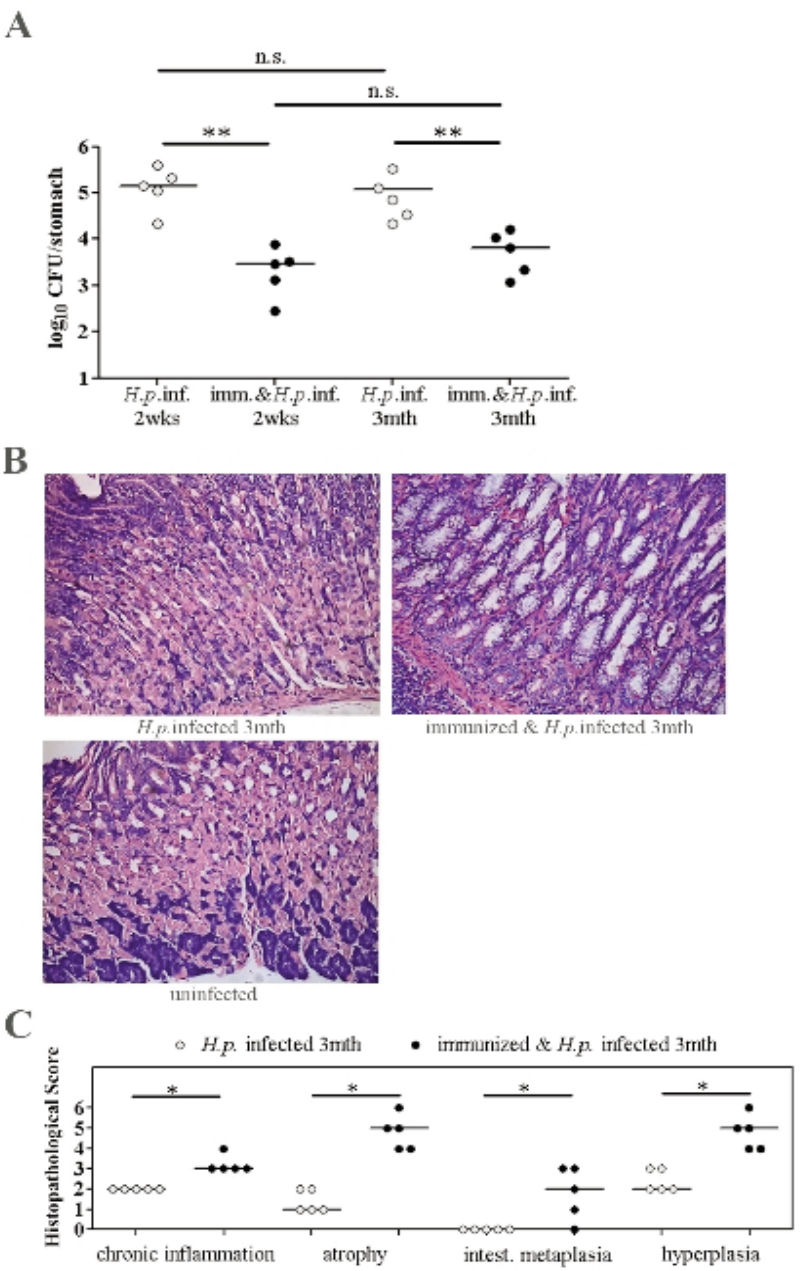


Figure 1. Mice that have been vaccinated against *Helicobacter pylori* develop more severe preneoplastic gastric pathology than non-immunized, infected controls. A group of six week old C57Bl6 mice were immunized with four consecutive weekly doses of 1mg *Helicobacter pylori* lysate plus 10µg cholera toxin, and challenged with live bacteria of the autologous strain two weeks after the last dose. The mice were sacrificed three months after the challenge infection, and were analyzed with respect to bacterial colonization (A) and gastric histopathology (B, C) in comparison to a control group that had been infected (without prior immunization) for three months with the same strain. Colonization levels were determined by plating of gastric homogenates and colony counting; gastric histopathology (assessing the parameters chronic inflammation, atrophy, epithelial hyperplasia, and intestinal metaplasia) was scored based on the criteria specified by the Sydney system on a scale of 0-6 (Sayi et al., 2009).

as IL-10 (Ismail et al., 2003) clear an experimental infection even if they have not been immunized prior to challenge, indicating that Tregs are crucial in balancing immunity and immunopathology in the *Helicobacter*-host interaction.

Having determined a critical role for IFN-γ in both clearance and pathology, we sought to identify the cell type producing the lion's share of the cytokine in the gastric mucosal environment. All T-cell subsets in addition to NK and NKT cells are known sources of IFN-γ. Adoptive transfer experiments using as few as 50,000 cells clearly showed that gastric IFN-γ production as well as the control of *Helicobacter* densities and the induction of gastric pathology could all be restored in TCR-$\beta^{-/-}$ and Rag-$1^{-/-}$ mice by intravenous injection of highly purified populations of $CD4^+CD25^-$ effector T-cells (Sayi et al., 2009). $CD8^+$ T-cells had no effect, even at 40 times higher numbers. It was also clear that no other sources of IFN-γ could substitute for $CD4^+CD25^-$ T-cells in the stomach in their absence. Clearance of *H. pylori* and gastric pathology was only observed if the adoptively transferred cells originated from an infected wild type donor; similarly, gastric pathology was only induced in infected recipients. Interestingly, $CD4^+CD25^-$ T-cells isolated from IFN-$\gamma^{-/-}$ donors were significantly less pathogenic and completely incapable of controlling the infection, indicating that this cell type is indeed crucial for *Helicobacter* clearance and also largely responsible for gastric preneoplastic changes. The adoptive transfer model allowed us to address which effector mechanisms might ultimately be required for efficient elimination of the bacteria from infected stomachs. As neutrophils had been implicated before in another non-vaccine-induced *Helicobacter* clearance model (Ismail et al., 2003), and IFN-γ is known to stimulate their phagocytic and microbicidal activities (Rodrigues et al., 2007; Yoshimura and Takahashi, 2007), we depleted neutrophils from the recipients of adoptively transferred $CD4^+CD25^-$ effector T-cells. Indeed, clearance of *Helicobacter* depended entirely on neutrophils, implying that these cells are ultimately needed to eliminate this extracellular, luminal bacterium.

The molecular mechanisms underlying the transformation of the normal gastric epithelium with its terminally differentiated, functionally specialized cells into predominantly hyperplastic or metaplastic tissue are largely unknown. To delineate a potential direct effect of IFN-γ on gastric epithelial cells, we generated an SV40-immortalized cell line from primary murine gastric cells with characteristics of mucus producing pit cells. Treatment of these immortalized cells with increasing doses of recombinant IFN-γ resulted in the up-regulation of numerous genes as determined by transcriptional profiling using whole genome microarrays. Many of the identified IFN-γ-responsive genes are known target genes of the IFN-γ signaling pathway. In parallel, we generated expression profiles of whole stomach mucosa consisting predominantly of metaplastic or hyperplastic cells and compared these to profiles of normal epithelium. Interestingly, the changes induced by IFN-γ *in vitro* paralleled those induced during the transformation from normal to hyperplastic, but not metaplastic mucosa, suggesting that IFN-γ acts *in vivo* to induce hyperproliferation of undifferentiated progenitors of the mucus producing lineages.

Other functionally related, T-cell-derived cytokines have also recently been implicated in the control of *Helicobacter* infection and the resulting immunopathology. Interleukin-17 (IL-17), a product of the newly identified $CD4^+$ subset of Th17 cells, is clearly up-regulated in the gastric mucosa of infected individuals (Caruso et al., 2007) and in experimentally infected mice (Shiomi et al., 2008). Two studies have identified a role for IL-17 in vaccine-induced protection, where it presumably serves to recruit neutrophils to the site of infection (IL-17 is known to induce the secretion of several neutrophil chemoattractants by epithelial cells; DeLyria et al., 2009; Velin et al., 2009); however, the issue of IL-17 contribution will only be resolved fully once vaccination data from knockout mice lacking either the cytokine or its receptor are available. Interestingly, *Helicobacter* colonization levels in experimentally infected, non-immunized IL-$17^{-/-}$ mice were lower than in wild type controls, ruling out an important contribution of this cytokine to the control of natural infection with the bacterium (Shiomi et al., 2008). Another inflammatory cytokine with confirmed ties to gastric *Helicobacter*-induced pathology is IL-1β. Polymorphisms in the *IL-1β* promoter affecting the baseline expression levels of the cytokine are associated with an elevated gastric cancer risk (El-Omar et al., 2000). Moreover, its transgenic overexpression under a stomach specific promoter is

sufficient to induce gastritis and even gastric cancer in the absence of infection (Tu et al., 2008). Since the expression of IL-1β depends on the Th1 master transcriptional regulator T-bet (Stoicov et al., 2009), IL-1β may in fact be a, direct or indirect, downstream effector of IFN-γ and Th1 cells (IFN-γ and IL-12 synergize in the induction of T-bet and in Th1 polarization of antigen-activated T-cells).

Taken together, most available data suggest that gastric cancer is a consequence of chronic inflammation, with $CD4^+$ IFN-γ-secreting, pathogenic T-cells causing the transformation from normal mucosa to hyperplastic and metaplastic lesions. According to this model, the function of the bacteria lies exclusively in initiating inflammation; bacterial toxins and other secreted (virulence) factors may have little or no direct role (other than modulating inflammation) in gastric neoplasia. Indeed, mouse strains that lack T-cells altogether are entirely protected from gastric cancer despite bacterial burdens that are easily ten times higher than those of wild type mice. The inseparable functions of T-cells in the protection against *Helicobacter* and in the pathogenesis of gastric neoplasia presents a tremendous challenge for vaccine development; any vaccine that fails to afford sterilizing immunity will likely increase rather than reduce gastric cancer risk.

References

Amieva MR, Salama NR, Tompkins LS, Falkow S. *Helicobacter pylori* enter and survive within multivesicular vacuoles of epithelial cells. *Cell Microbiol* 4:677-690, 2002.

Caruso R, Pallone F, Monteleone G. Emerging role of IL-23/IL-17 axis in *H. pylori*-associated pathology. *World J Gastroenterol* 13:5547-5551, 2007.

Correa P. A human model of gastric carcinogenesis. *Cancer Res* 48:3554-3560, 1988.

Correa P. *Helicobacter pylori* and gastric carcinogenesis. *Am J Surg Pathol* 19(Suppl 1):S37-43, 1995.

Correa P, Haenszel W, Cuello C, Tannenbaum S, Archer M. A model for gastric cancer epidemiology. *Lancet* 2:58-60, 1975.

DeLyria ES, Redline RW, Blanchard TG. Vaccination of mice against *H. pylori* induces a strong Th-17 response and immunity that is neutrophil dependent. *Gastroenterology* 136:247-256, 2009.

El-Omar EM, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N, Lanyon G, Martin M, Fraumeni JF, Jr., Rabkin CS. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature* 404:398-402, 2000.

Fox JG, Beck P, Dangler CA, Whary MT, Wang TC, Shi HN, Nagler-Anderson C. Concurrent enteric helminth infection modulates inflammation and gastric immune responses and reduces *Helicobacter*-induced gastric atrophy. *Nat Med* 6:536-542, 2000.

Fox JG, Rogers AB, Ihrig M, Taylor NS, Whary MT, Dockray G, Varro A, Wang TC. *Helicobacter pylori*-associated gastric cancer in INS-GAS mice is gender specific. *Cancer Res* 63:942-950, 2003a.

Fox JG, Sheppard BJ, Dangler CA, Whary MT, Ihrig M, Wang TC. Germ-line p53-targeted disruption inhibits *Helicobacter*-induced premalignant lesions and invasive gastric carcinoma through down-regulation of Th1 proinflammatory responses. *Cancer Res* 62:696-702, 2002.

Fox JG, Wang TC, Rogers AB, Poutahidis T, Ge Z, Taylor N, Dangler CA, Israel DA, Krishna U, Gaus K, Peek RM, Jr. Host and microbial constituents influence *Helicobacter pylori*-induced cancer in a murine model of hypergastrinemia. *Gastroenterology* 124:1879-1890, 2003b.

Ismail HF, Fick P, Zhang J, Lynch RG, Berg DJ. Depletion of neutrophils in IL-10(-/-) mice delays clearance of gastric *Helicobacter* infection and decreases the Th1 immune response to Helicobacter. *J Immunol* 170:3782-3789, 2003.

Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1:1311-1315, 1984.

Nystrom J, Svennerholm AM. Oral immunization with HpaA affords therapeutic protective immunity against *H. pylori* that is reflected by specific mucosal immune responses. *Vaccine* 25:2591-

2598, 2007.

Oh JD, Karam SM, Gordon JI. Intracellular *Helicobacter pylori* in gastric epithelial progenitors. *Proc Natl Acad Sci USA* 102:5186-5191, 2005.

Parsonnet J, Friedman GD, Orentreich N, Vogelman H. Risk for gastric cancer in people with CagA positive or CagA negative *Helicobacter pylori* infection. *Gut* 40:297-301, 1997.

Parsonnet J, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, Sibley RK. *Helicobacter pylori* infection and the risk of gastric carcinoma. *N Engl J Med* 325:1127-1131, 1991.

Parsonnet J, Hansen S, Rodriguez L, Gelb AB, Warnke RA, Jellum E, Orentreich N, Vogelman JH, Friedman GD. *Helicobacter pylori* infection and gastric lymphoma. *N Engl J Med* 330:1267-1271, 1994.

Rodrigues DR, Dias-Melicio LA, Calvi SA, Peracoli MT, Soares AM. *Paracoccidioides brasiliensis* killing by IFN-gamma, TNF-alpha and GM-CSF activated human neutrophils: role for oxygen metabolites. *Med Mycol* 45:27-33, 2007.

Sayi A, Kohler E, Hitzler I, Arnold I, Schwendener R, Rehrauer H, Muller A. The CD4+ T cell-mediated IFN-gamma response to *Helicobacter* infection is essential for clearance and determines gastric cancer risk. *J Immunol* 182:7085-7101, 2009.

Shiomi S, Toriie A, Imamura S, Konishi H, Mitsufuji S, Iwakura Y, Yamaoka Y, Ota H, Yamamoto T, Imanishi J, Kita M. IL-17 is involved in *Helicobacter pylori*-induced gastric inflammatory responses in a mouse model. *Helicobacter* 13:518-524, 2008.

Skene CD, Doidge C, Sutton P. Evaluation of ISCOMATRIX and ISCOM vaccines for immunisation against *Helicobacter pylori*. *Vaccine* 26:3880-3884, 2008.

Stoicov C, Fan X, Liu JH, Bowen G, Whary M, Kurt-Jones E, Houghton J. T-bet knockout prevents *Helicobacter felis*-induced gastric cancer. *J Immunol* 183:642-649, 2009.

Tu S, Bhagat G, Cui G, Takaishi S, Kurt-Jones EA, Rickman B, Betz KS, Penz-Oesterreicher M, Bjorkdahl O, Fox JG, Wang TC. Overexpression of interleukin-1beta induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. *Cancer Cell* 14:408-419, 2008.

Velin D, Favre L, Bernasconi E, Bachmann D, Pythoud C, Saiji E, Bouzourene H, Michetti P. Interleukin-17 is a critical mediator of vaccine-induced reduction of *Helicobacter* infection in the mouse model. *Gastroenterology* 136:2237-2246, 2009.

Wiedemann T, Loell E, Mueller S, Stoeckelhuber M, Stolte M, Haas R, Rieder G. *Helicobacter pylori* cag-Pathogenicity island-dependent early immunological response triggers later precancerous gastric changes in Mongolian gerbils. *PLoS One* 4:e4754, 2009.

Yoshimura T, Takahashi M. IFN-gamma-mediated survival enables human neutrophils to produce MCP-1/CCL2 in response to activation by TLR ligands. *J Immunol* 179:1942-1949, 2007.

Zhang M, Berndt BE, Eaton KA, Rathinavelu S, Pierzchala A, Kao JY. *Helicobacter pylori*-pulsed dendritic cells induce H. pylori-specific immunity in mice. *Helicobacter* 13:200-208, 2008.

3.3 Dendritic cells prevent rather than promote immunity conferred by a *Helicobacter* vaccine using a mycobacterial adjuvant

article in press, Gastroenterology, 2011

authors: Iris Hitzler, Mathias Oertli, Burkhard Becher, Else-Marie Agger and Anne Müller

contributions: I designed, conducted and analyzed all experiments except figure 6E-F (MO) and wrote the manuscript together with AM. BB and EMA contributed vital tools.

Dendritic Cells Prevent Rather Than Promote Immunity Conferred by a *Helicobacter* Vaccine Using a Mycobacterial Adjuvant

IRIS HITZLER,* MATHIAS OERTLI,* BURKHARD BECHER,‡ ELSE MARIE AGGER,§ and ANNE MÜLLER*

*Institute of Molecular Cancer Research and ‡Institute of Experimental Immunology, University of Zurich, Zurich, Switzerland; §Department of Infectious Disease Immunology, Statens Serum Institut, Copenhagen, Denmark

BACKGROUND & AIMS: Immunization against the gastric bacterium *Helicobacter pylori* could prevent many gastric cancers and other disorders. Most vaccination protocols used in preclinical models are not suitable for humans. New adjuvants and a better understanding of the correlates and requirements for vaccine-induced protection are needed to accelerate development of vaccines for *H pylori*. **METHODS:** Vaccine-induced protection against *H pylori* infection and its local and systemic immunological correlates were assessed in animal models, using cholera toxin or CAF01 as adjuvants. The contribution of B cells, T-helper (Th)–cell subsets, and dendritic cells to *H pylori*–specific protection were analyzed in mice. **RESULTS:** Parenteral administration of a whole-cell sonicate, combined with the mycobacterial cell-wall–derived adjuvant CAF01, protected against infection with *H pylori* and required cell-mediated, but not humoral, immunity. The vaccine-induced control of *H pylori* was accompanied by Th1 and Th17 responses in the gastric mucosa and in the gut-draining mesenteric lymph nodes; both Th subsets were required for protective immunity against *H pylori*. The numbers of memory $CD4^+$ T cells and neutrophils in gastric tissue were identified as the best correlates of protection. Systemic depletion of dendritic cells or regulatory T cells during challenge infection significantly increased protection by overriding immunological tolerance mechanisms activated by live *H pylori*. **CONCLUSIONS: Parenteral immunization with a *Helicobacter* vaccine using a novel mycobacterial adjuvant induces protective immunity against *H pylori* that is mediated by Th1 and Th17 cells. Tolerance mechanisms mediated by dendritic cells and regulatory T cells impair *H pylori* clearance and must be overcome to improve immunity.**

Keywords: Gastric Cancer; Bacteria; Ulcer; Gastric Adenocarcinoma.

BASIC AND TRANSLATIONAL AT

Helicobacter pylori colonizes half of the world's population and chronic gastric infection with this bacterium leads to the development of gastric ulcers, gastric adenocarcinoma, or mucosa-associated lymphoid tissue lymphoma in 1%–10% of infected individuals.[1] The standard *H pylori* eradication therapy consists of 2 to 3 antibiotics and an acid suppressant; however, antibiotic resistance rates are rising,[2] a subset of individuals relapse even after efficient eradication,[3] and the infection is often not diagnosed until gastric premalignant lesions have progressed to an irreversible stage.[4] Immunization against *H pylori,* therefore, represents an attractive alternative strategy for the prevention of gastric cancer and other *Helicobacter*-associated gastric disorders.

No vaccine regimens are currently available for human use. The gold standard vaccination protocol that has been used extensively in preclinical models combines the mucosal adjuvant cholera toxin (CT) with whole-cell preparations of *H pylori* or *Helicobacter felis* into orally administered formulations.[5–8] Because of the toxicity of CT, human trials have bypassed this effective adjuvant in favor of other less toxic compounds, such as the heat-labile enterotoxin of *Escherichia coli*, which was combined with recombinant urease B- or whole-cell preparations to generate well-tolerated, but largely ineffective formulations.[9–11] A live recombinant *Salmonella enterica* serovar Typhi Ty21a vaccine expressing *H pylori*'s urease A and B subunits also did not show sufficient immunogenicity in human volunteers,[12,13] but confirmed a direct correlation between *H pylori*–specific T-cell responses and bacterial clearance.[14]

Protective immunity against *H pylori* can be achieved by either Th1 and/or Th17-polarizing adjuvants[5,15–18] or by aluminum-based adjuvants that stimulate Th2-polarized responses.[19,20] Both complete Freund's adjuvant and alum have been used successfully in preventive[20,21] and even therapeutic[19] vaccination strategies. An alum-adjuvanted parenteral vaccine using the *H pylori* antigens VacA, CagA, and NAP was recently reported to be immunogenic and safe in humans.[22] Here we focus on a novel adjuvant for Th1/Th17 vaccination that incorporates a synthetic analog (trehalose-6,6-dibehenate) of the mycobacterial cell wall glycolipid trehalose-6,6-dimycolate into a cationic liposome-based adjuvant formulation (CAF01).[23] CAF01 facilitates antigen uptake and presentation by dendritic cells (DCs) and macrophages[24,25] and confers protective immunity to a variety of infectious agents, including *Mycobacterium tuberculosis, Chlamydia muridarum,* and the malarial parasite *Plasmodium yoelii.*[26,27] The mixed Th1/Th17

Abbreviations used in this paper: APC, allophycocyanin; CT, cholera toxin; DC, dendritic cell; DT, diphtheria toxin; IFN-γ, interferon-γ; IL, interleukin; IP, intraperitoneal; MHC, major histocompatibility complex; MLN, mesenteric lymph nodes; PB, pacific blue; SC, subcutaneous; Th1/2/17, T-helper type 1/2/17; Treg, regulatory T cell.

0016-5085/$36.00
doi:10.1053/j.gastro.2011.04.009

T-cell responses elicited by CAF01-adjuvanted vaccination have been particularly well-characterized in the context of *M tuberculosis* infection.[23]

Here, we compared various routes of administration of a CAF01-adjuvanted *H pylori* whole-cell vaccine to a CT-adjuvanted immunization protocol with respect to protection from autologous challenge infection. We show that parenteral—but not mucosal—immunization with CAF01 elicits protective immunity to *H pylori* challenge infection that is antibody-independent, but requires both Th1 and Th17 subsets. Other correlates of protection included gastric memory T-cell, neutrophil, and mast cell infiltration. We further report here that immunoregulatory mechanisms involving DCs and regulatory T cells (Treg) impair protective immunity; consequently, the systemic depletion of DCs or Treg during challenge infection significantly improved the efficacy of *H pylori*–specific vaccination.

Materials and Methods

Animal Experimentation

C57BL/6 wild-type, major histocompatibility complex (MHC) $II^{-/-}$, $JHT^{-/-}$ (which lack J_H segments and the intron enhancer in the IgH locus and therefore cannot generate B cells),[28] interleukin (IL)-12 $p35^{-/-}$, IL-12/IL-23 $p40^{-/-}$, and *CD11c*-DTR tg mice (expressing the diphtheria toxin [DT] receptor under the control of the *CD11c* promoter) were originally purchased from Charles River Laboratories (Wilmington, MA). *FoxP3*-EGFP-DTR tg mice[29] and IL-23 $p19^{-/-}$ mice were kindly provided by Tim Sparwasser and Regeneron Pharmaceuticals, respectively. All mice were bred at a University of Zurich specific pathogen-free facility. Mixed-sex groups were included in studies at 6 weeks of age. All animal experimentation was reviewed and approved by the Zurich cantonal veterinary office. Mice were immunized 3 times at weekly intervals. A stable CAF01 formulation consisting of dimethyldioctadecylammonium bromide and a,a′-trehalose 6,6′-dibehenate (Avanti Polar Lipids, Alabaster, AL) was prepared by the lipid film hydration method, as described previously.[26] For CAF01 vaccinations, 250 μg *H pylori* SS1[30] whole-cell sonicate was given per dose. In the case of intraperitoneal (IP), oral, or subcutaneous (SC) immunization at the base of the neck, the total volume was 200 μL. For intranasal immunizations, a total volume of 50 μL was given in drops of 12.5 μL to alternating nostrils of mice anesthetized with isoflurane (Minrad, Buffalo, NY). For CT immunization, mice were administered 500 μg sonicate along with 10 μg CT (List Biologicals, Campbell, CA) by oral gavage. Two weeks after the last immunization, immunized and naïve mice were infected with 10^8 *H pylori* SS1 (colony-forming unit estimated based on optical density) grown as described previously.[7] Mice were sacrificed 2 weeks after challenge. Bacterial colonization was assessed by colony count assay as described earlier.[7] DT (Sigma-Aldrich, St Louis, MO) was injected IP every 2 days for the depletion of DCs (4 ng/g body weight) or of Treg (40 ng/g body weight).

Preparation of Gastric and Mesenteric Lymph Node Single-Cell Suspensions, Flow Cytometry, Enzyme-Linked Immunosorbent Assay, Quantitative Reverse Transcription Polymerase Chain Reaction, and Treg Conversion Assay

One-sixth of every stomach (antrum and corpus) and corresponding mesenteric lymph nodes were digested in 1 mg/mL collagenase (Sigma-Aldrich) for 45 minutes at 37°C with shaking before mechanical disruption between glass slides and filtering. Single-cell suspensions were either stained directly for flow cytometric analysis or seeded at 200-k cells/well into 96-well round-bottom plates. The following antibodies were used: CD4–fluorescein isothiocyanate, CD4-allophycocyanin (APC) (BD Biosciences, San Diego, CA), CD45-pacific blue (PB), Ly6G-APC, CD62L-APC, CD44-PB, rat anti-mouse CD117 (all BioLegend, San Diego, CA), followed by goat anti-rat fluorescein isothiocyanate (Sigma-Aldrich) and CD11c-biotin (BD Biosciences), followed by streptavidin-PB (Life Technologies, Carlsbad, CA). Interferon-γ (IFN-γ)-phycoerythrin-Cy7 (BD), IL-17–APC, and FoxP3-APC (all eBioscience, San Diego, CA) were used for intracellular staining. Before intracellular cytokine staining, cells were stimulated and blocked in 2.5 μg/mL Brefeldin A (AppliChem, Darmstadt, Germany), 0.2 μM ionomycin (Santa Cruz Biotechnology, Santa Cruz, CA), and 50 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich) for 5 hours, stained for extracellular markers, and fixed in 4% paraformaldehyde. Flow cytometry was performed on a Cyan ADP 9 instrument (Beckman Coulter, Brea, CA) and analyzed using FlowJo software (TreeStar, Ashland, OR). Enzyme-linked immunosorbent assay and quantitative reverse transcription polymerase chain reaction techniques, as well as the protocols for Treg conversion are described in the Supplementary Methods.

Statistics

GraphPad Prism (GraphPad Software, La Jolla, CA) was used for statistical analyses. Colonization counts were compared by Mann–Whitney test. All other indicated *P* values were calculated by Student *t* test.

Results

Parenteral CAF01-Adjuvanted Vaccination Confers Protective Immunity to H pylori

To test the efficacy of the synthetic mycobacterial adjuvant CAF01 in *H pylori*–specific vaccination, we immunized mice with either the gold standard CT-adjuvanted, orally administered *H pylori* sonicate vaccine, or mixed the same sonicate with CAF01 for SC, IP, intranasal, and oral administration. Two weeks after the last of 3 weekly doses, all immunized mice as well as nonimmunized controls were challenged with 10^8 live *H pylori* SS1 and sacrificed 2 weeks later (see timetable in Figure 1*A*).

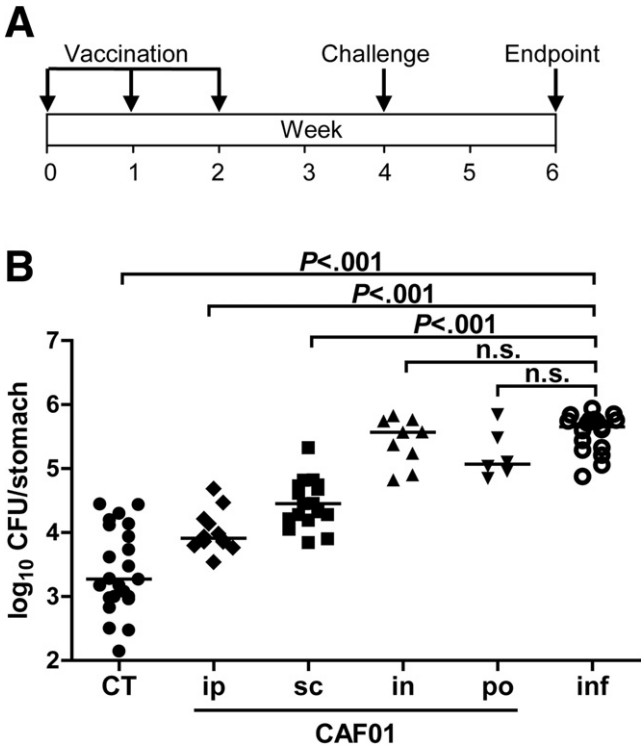


Figure 1. CAF01 adjuvant elicits protective immunity against *H pylori*. (*A*) Timetable of CAF01 and CT immunizations. (*B*) *H pylori* colonization levels as assessed by colony count assay (colony-forming units) for mice vaccinated orally with CT or with CAF01 adjuvant via different routes (ip, intraperitoneal; sc, subcutaneous; in, intranasal; po, oral) as well as for infected controls (inf). *Horizontal lines* represent median values. Combined data from 2 to 4 independent studies are shown.

The CAF01-adjuvanted vaccine provided protective immunity when administered parenterally (SC or IP), but not when given via mucosal routes (ie, intranasal or oral; Figure 1*B*). Speculating that the challenge dose might influence protection, we infected groups of SC CAF01-immunized mice with challenge doses ranging from 10^3 to 10^9 *H pylori*. Interestingly, all challenged mice were protected to the same degree, arguing that even minute *H pylori* burdens cannot be cleared completely by CAF01-vaccinated hosts (Supplementary Figure 1*A*). Assessing vaccine efficacy at a later time point post challenge (5 weeks) did not affect the vaccination outcome (Supplementary Figure 1*B*). The combined results suggest that the novel adjuvant CAF01 shows efficacy in *H pylori*–specific systemic vaccination strategies.

Th1-Mediated Immunity Is Associated With CAF01-Induced Protection Against H pylori

In order to identify immunological correlates of CAF01 vaccine-mediated protective immunity to *H pylori*, we examined *H pylori*–specific serum and mucosal antibody titers in the treatment arms outlined here. Most immunization strategies (except oral vaccination with CAF01) induced significantly elevated levels of *H pylori*–specific serum and mucosal IgG but not IgA; the mucosal IgG responses were generally more predictive of vaccination outcome than the serum IgG responses (Figure 2*A–C*). As relative IgG2c and IgG1 levels serve as indicators of systemic Th1 and Th2 polarization of $CD4^+$ Th cells, we separately quantified both IgG subclasses and calculated their ratios. Interestingly, only the IP- or SC-immunized, protected mice produced significant amounts of serum and mucosal IgG2c, whereas IgG1 was also generated by unprotected mice (Figure 2*D, E*), indicating that the Th1 polarization of activated T cells is a correlate and possible prerequisite for protection. To examine this further, we compared gastric levels of the Th1 and Th17 signature cytokines IFN-γ and IL-17 in the various treatment arms. Indeed, mice that were well-protected, ie, the CT- as well as the IP and SC CAF-immunized groups showed a significantly higher level of IFN-γ and IL-17 expression at the site of challenge infection than unprotected mice or infected-only controls (Figure 2*F*). Overall, these data imply that indicators of Th1/Th17 polarization detected either locally or systemically represent useful correlates of protective immunity to *H pylori*.

Antibodies Are Dispensable and $CD4^+$ T Cells Are Required for CAF01-Mediated Protective Immunity

Previous studies have shown that *H pylori*–specific antibodies may be generated in vaccinated/challenged animals, but are not necessarily required for protection.[31–33] In order to clarify this issue in the context of our vaccination strategies, we immunized $JHT^{-/-}$ mice lacking functional B cells with either the CT- or the CAF01-adjuvanted vaccine (Figure 3*A*, *left* and *right panels*). Both groups of vaccinated mice controlled the challenge infection as well as wild-type animals (Figure 3*A*), indicating that neither B cells nor *H pylori*–specific antibodies are required for protection. In contrast, mice lacking a functional $CD4^+$ T-cell compartment due to a targeted deletion of the MHC class II locus were incapable of controlling the challenge infection in both immunization arms of the study (Figure 3*B*). Therefore, B cells are dispensable and MHC class II–restricted T cells are strictly required for *H pylori*–specific immunity in these models.

Immune Cell Infiltration Into the Stomach Correlates With the Level of Protection

To identify additional correlates and a possible mechanism of vaccine-induced protection, we characterized the immune cell populations infiltrating the gastric mucosa in a panel of CT- or CAF01-immunized mice compared to control-infected mice. The protection levels achieved by the various treatment arms were representative in this study (Figure 4*A*). Whole-stomach, single-cell preparations were generated for individual mice and analyzed flow cytometrically with respect to the infiltration of $CD45^+$ leukocytes (Figure 4*B*), $CD4^+$ T cells (Figure 4*C*), and $CD4^+CD62L^-CD44^+$ activated memory T cells (Figure 4*D*). All 3 populations were elevated in those treatment arms that had successfully controlled the challenge infection, but not in the unprotected mice (Figure

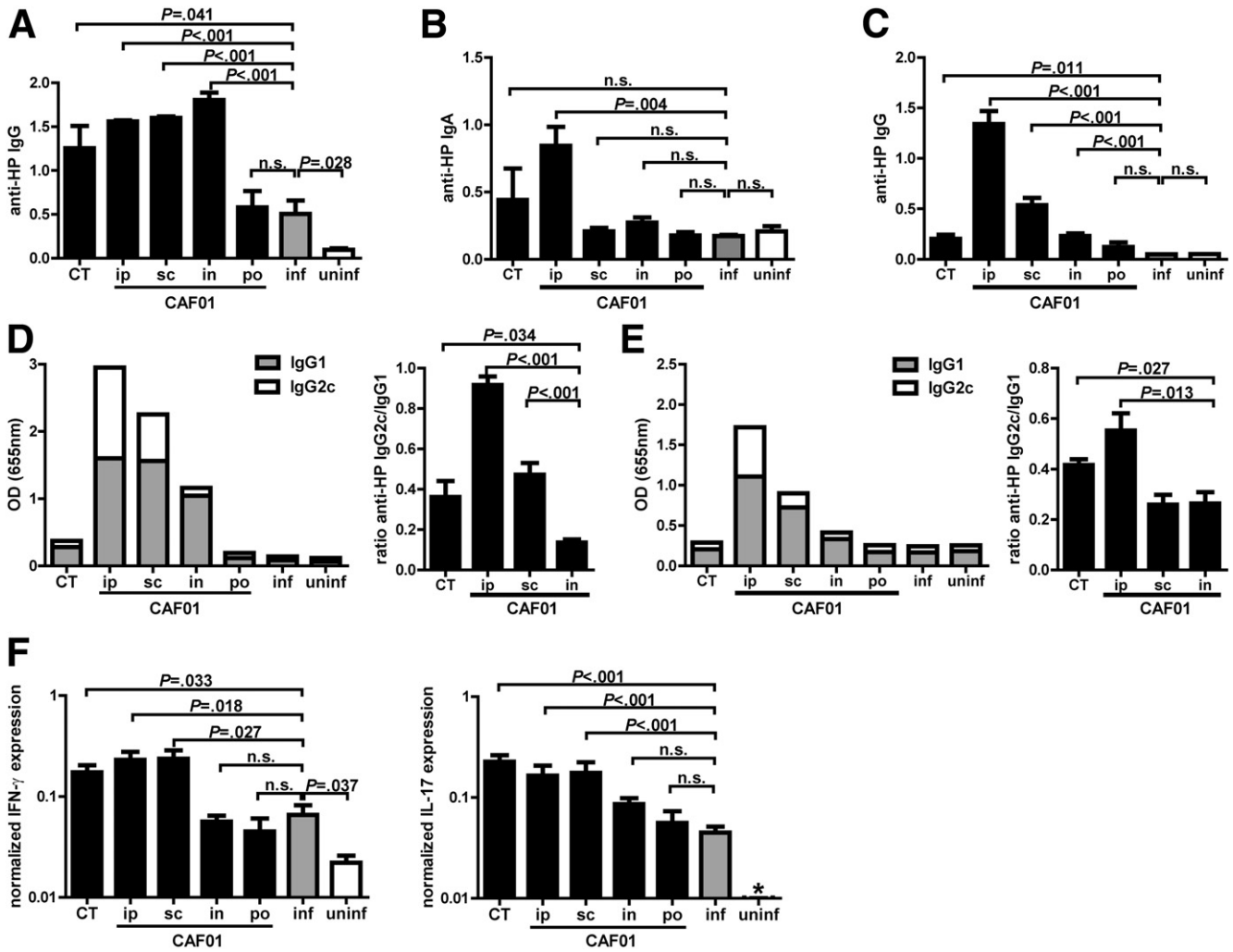


Figure 2. Protection against *H pylori* (HP) requires Th1-biased immunity. (*A–C*) Relative levels of *H pylori*–specific serum IgG (*A*), mucosal IgA (*B*), and mucosal IgG (*C*) in immunized, infected, and uninfected mice, measured by enzyme-linked immunosorbent assay and represented as optical density values. (*D*) Serum and (*E*) mucosal *H pylori*–specific IgG1 and IgG2c levels of the same mice (*left panels*), as well as IgG2c/IgG1 ratios (*right panels*) for mice with antibody levels above baseline. (*F*) Gastric IFN-γ and IL-17 transcript levels as determined by real-time reverse transcription polymerase chain reaction, normalized to glyceraldehyde-3-phosphate dehydrogenase expression. Groups of 4–10 animals were examined in *panels A–F*; data are shown as mean ± SEM. *Not detectable.

4*B–D*). In line with previously published data implicating Ly6-G$^+$ neutrophils and c-Kit$^+$ mast cells in the clearance of *H pylori,*[5,34] both populations were enriched in the mucosa of protected but not unprotected mice (Figure 4*E, F*). Overall, these data suggest that the gastric infiltration of immune cells, in particular of memory T cells, leukocytes, and neutrophils (and to a lesser extent of mast cells), serves as a reliable marker of protection against *H pylori* infection and highlights the importance of local mucosal immune responses in the control of this extracellular, luminal pathogen.

CAF01-Adjuvanted Vaccination Elicits a Mixed Th1/Th17 Response Upon Challenge Infection; Both Th Subsets Are Required for Protective Immunity Against H pylori

Th1 cells as well as Th17 cells have been implicated in the control of *H pylori* infection after CT-adjuvanted vaccination[5,18] and CAF01 is known to induce mixed Th1 and Th17 responses.[23] Therefore, we characterized the cytokine expression profiles of CD4$^+$ T cells infiltrating the stomachs of CAF01-immunized mice after challenge infection. Intracellular cytokine staining for IFN-γ and IL-17 revealed that the numbers of both stomach-infiltrating IFN-γ$^+$ CD4$^+$ T cells and IL-17$^+$ CD4$^+$ T cells were significantly higher in CAF01-immunized, challenged mice than in infected-only controls; infected mice in turn exhibited higher numbers of cytokine-positive cells than uninfected controls (Figure 5*A*). Having shown earlier that the gut-draining mesenteric lymph nodes (MLN) are important sites of *H pylori*–specific T-cell priming,[35] we hypothesized that similar differences in IFN-γ– and IL-17–producing T cells should be detectable in single-cell MLN preparations. We cultured MLN suspensions of individual mice for 4 days before restimulation and intracellular cytokine staining for IFN-γ and IL-17. Again, significantly more cytokine-expressing T cells of both

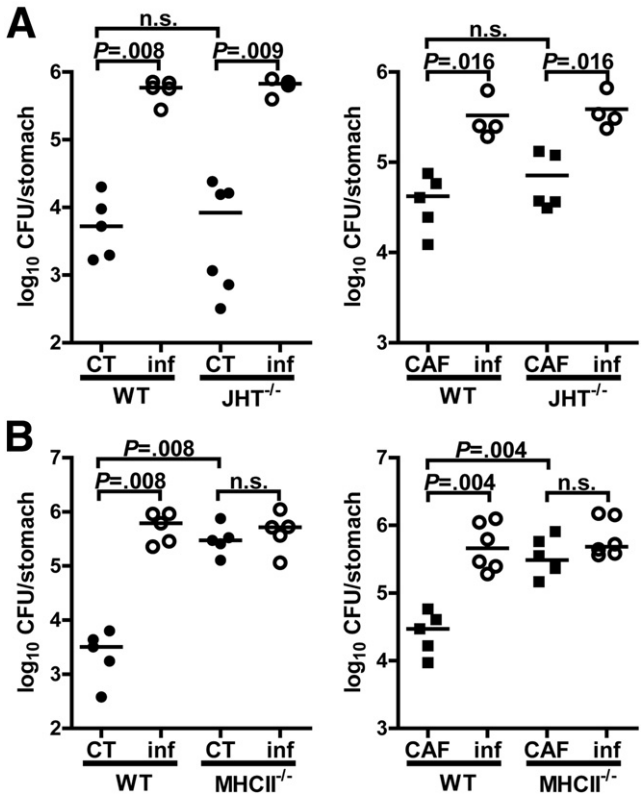


Figure 3. T cells but not B cells are required for CAF01-induced protection. (*A–B*) Colonization levels of (*A*) JHT$^{-/-}$ and (*B*) MHC class II$^{-/-}$ mice that were vaccinated with either CT (*left panels*) or CAF01 SC (*right panels*) or remained unvaccinated (inf) in comparison to wild-type mice.

populations were detected in the CAF01-vaccinated compared to infected-only mice, which in turn exhibited higher numbers than uninfected controls (Figure 5*B*). Further experiments confirmed that IFN-γ and IL-17 produced in the MLN of vaccinated mice were indeed derived from *H pylori*–specific T cells (Supplementary Figure 2). The combined observations suggest that mixed Th1/Th17 responses are a general hallmark of protective immunity to *H pylori*. To clarify whether both Th subsets are essential for vaccine-induced protection, we immunized IL-23p19$^{-/-}$ mice (which fail to generate Th17 cells), IL-12p35$^{-/-}$ mice (no Th1 cells), and IL-12/23p40$^{-/-}$ mice (neither Th1 nor Th17 cells) with our CAF-adjuvanted vaccine before challenge infection. Neither IL-23p19$^{-/-}$ nor IL-12p35$^{-/-}$ mice nor IL-12/23p40$^{-/-}$ mice were able to control the challenge infection as determined by colony counting (Figure 5*C*); all protection correlates (leukocyte, memory T cell, mast cell, neutrophil infiltration) confirmed the defect in developing protective immunity of the 3 strains (Figure 5*D*). Intracellular cytokine staining and quantitative polymerase chain reaction revealed that the gastric infiltration of Th1 cells and gastric IFN-γ production were reduced as expected in the IL-12p35$^{-/-}$ mice and IL-12/23p40$^{-/-}$ mice (and somewhat also in the IL-23p19$^{-/-}$ mice), whereas the gastric numbers of Th17 cells and gastric IL-17 production were reduced in the IL-23p19$^{-/-}$ and IL-12/23p40$^{-/-}$ mice (Figure 5*E, F*). IL-12/23p40$^{-/-}$ mice showed an additive effect with respect to most protection correlates (Figure 5*D–F*). In summary, we conclude that mixed Th1 and Th17 responses are hallmarks of protective immunity conferred by a CAF-adjuvanted *H pylori* vaccine, and that both Th responses are absolutely required for protection.

Regulatory T Cells Impair Protective Immunity to H pylori

None of the currently available vaccine regimens targeting *H pylori* induce sterilizing immunity; this conundrum is one of the main obstacles in *H pylori* vaccine development.[36] Hypothesizing that Tregs might actively counteract anti–*H pylori* immunity by favoring peripheral tolerance to the infection, we depleted FoxP3$^+$ Tregs systemically during the challenge phase of our study protocol in a strain expressing the DT receptor under the control of the *foxp3* promoter.[29] The depletion of Tregs (which was >80% efficient in the stomach as determined at the study end point, Supplementary Figure 3) strongly boosted *H pylori*–specific immunity as determined by lower colony counts (Figure 6*A*), higher gastric infiltration of leukocytes, memory T cells, mast cells, and neutrophils (Figure 6*B*) and higher numbers of gastric IFN-γ$^+$ and IL-17$^+$ T cells (Figure 6*C, D*). A more stringent control of the challenge infection was also observed in naïve mice upon depletion of Tregs (Figure 6*A–D*).

Peripheral tolerance to non–self-antigens in the gastrointestinal tract is induced and maintained by DCs; therefore, we speculated that DCs might have an important role in tolerance induction to *H pylori*. We characterized the tolerogenic/immunoregulatory potential of DCs from infected mice ex vivo by testing their ability to convert naïve CD4$^+$ T cells to FoxP3$^+$ Treg in a transforming growth factor–β-dependent manner. CD11c$^+$ cells were enriched by immunomagnetic sorting from single-cell MLN preparations of infected mice, uninfected controls, or immunized/infected mice. The resulting >80% pure DC preparations were then cocultured with splenic CD4$^+$CD25$^-$ T cells from naïve mice under conditions of CD3 activation and transforming growth factor–β and IL-2 exposure (Figure 6*E*). DCs from infected animals induced FoxP3$^+$ Treg more efficiently than DCs from uninfected controls or from immunized/infected mice (Figure 6*E*). The ex vivo–induced Treg further expressed high levels of IL-10 as assessed by intracellular cytokine staining (Figure 6*F*). In conclusion, the exposure of mice to live *H pylori* generates MLN DCs with tolerogenic properties that are very efficient inducers of Tregs; Tregs in turn actively inhibit *H pylori* clearance and their depletion enhances *H pylori*–specific protective immunity.

Depletion of DCs During H pylori *Challenge Improves Vaccine-Induced Protective Immunity*

As DCs are known to induce tolerance in the gastrointestinal tract and DCs from infected mice are more potent inducers of Tregs than DCs from uninfected

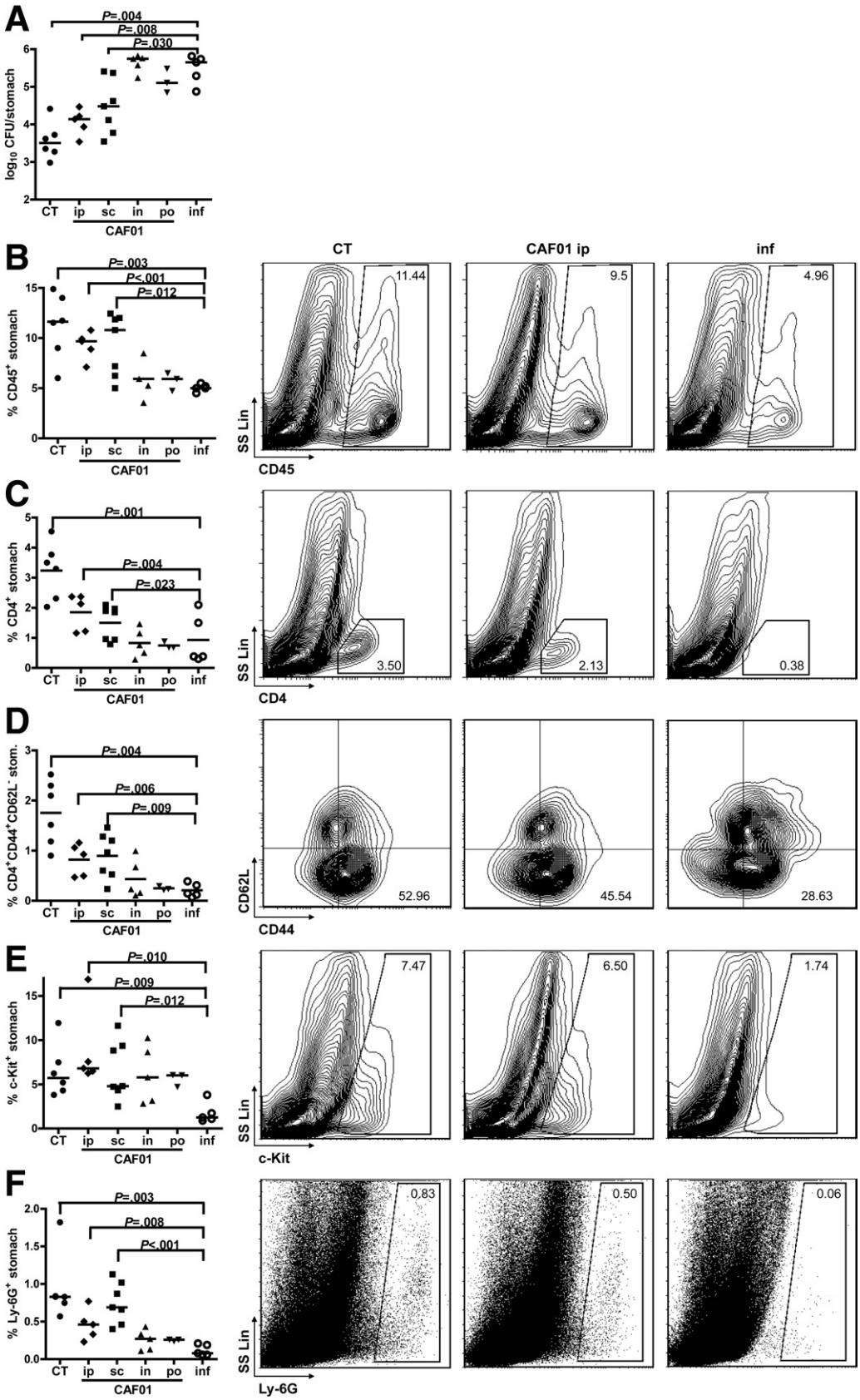


Figure 4. CAF01 vaccine-induced protection is accompanied by gastric infiltration of various immune cell types. (*A*) Colonization levels of the mice analyzed with respect to gastric infiltration in *panels B–F*. (*B–F*) Gastric immune cell infiltration in % of total stomach cells (*left panels*) and representative flow cytometry plots (*right panels*) of CT-immunized, CAF01-immunized, and infected-only (inf) mice. Leukocytes ($CD45^+$, *B*), $CD4^+$ T cells (*C*), activated memory $CD4^+$ T cells ($CD44^+CD62L^-$; *D*), mast cells (c-Kit^+; *E*), and neutrophils (Ly6-G^+; *F*) are shown. In *panel D*, the plots show the $CD44^+CD62L^-$ fraction of all gastric $CD4^+$ cells. *Panels A–F* show data from 2 combined experiments.

donors, we postulated that the systemic depletion of DCs during the challenge phase of our immunization protocol should improve vaccine-induced protection. To test this possibility, we immunized mice expressing the DT receptor under the control of the DC-specific *cd11c* promoter (*cd11c*-DTR tg) and depleted DCs systemically during the

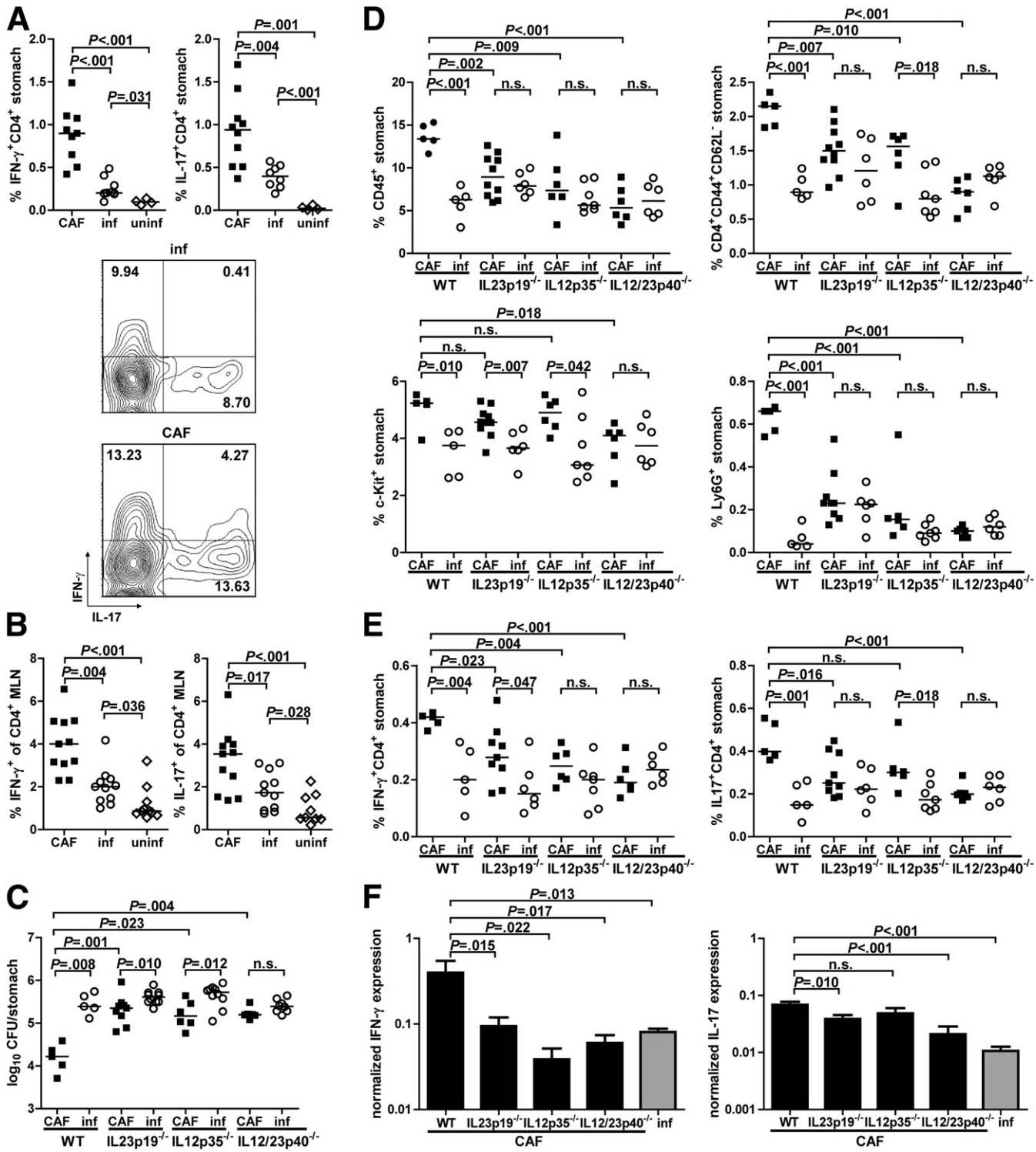


Figure 5. Mixed Th1/Th17 responses against *H pylori* are elicited by a CAF01-adjuvanted vaccine and both Th subsets are required for protection. (*A*) IFN-γ– and IL-17–producing CD4$^+$ T cells in % of all stomach cells in CAF01-immunized, infected, and uninfected wild-type mice as determined by intracellular cytokine staining (pooled data from 2 independent experiments; *upper panels*). Representative flow cytometry plots are also shown (*lower panels*). (*B*) IFN-γ– and IL-17–positive fractions of total CD4$^+$ T cells in MLN of CAF01-immunized, infected, and uninfected mice. (*C*) Colonization levels of wild-type, IL-23p19$^{-/-}$, IL-12p35$^{-/-}$, and IL-12/23p40$^{-/-}$ mice immunized SC with CAF01, relative to infected-only controls. (*D, E*) Gastric infiltration in percent of total stomach cells of leukocytes (CD45$^+$), activated memory CD4$^+$ T cells (CD44$^+$CD62L$^-$), mast cells (c-Kit$^+$), and neutrophils (Ly6-G$^+$), (*D*) as well as IFN-γ– and IL-17–producing CD4$^+$ T cells (*E*) of the mice shown in *panel C*. (*F*) Real-time reverse transcription polymerase chain reaction results, normalized to glyceraldehyde-3-phosphate dehydrogenase, for IFN-γ and IL-17 expression in immunized wild-type, IL-23p19$^{-/-}$, IL-12p35$^{-/-}$, and IL-12/23p40$^{-/-}$ mice relative to infected-only (inf) wild-type controls.

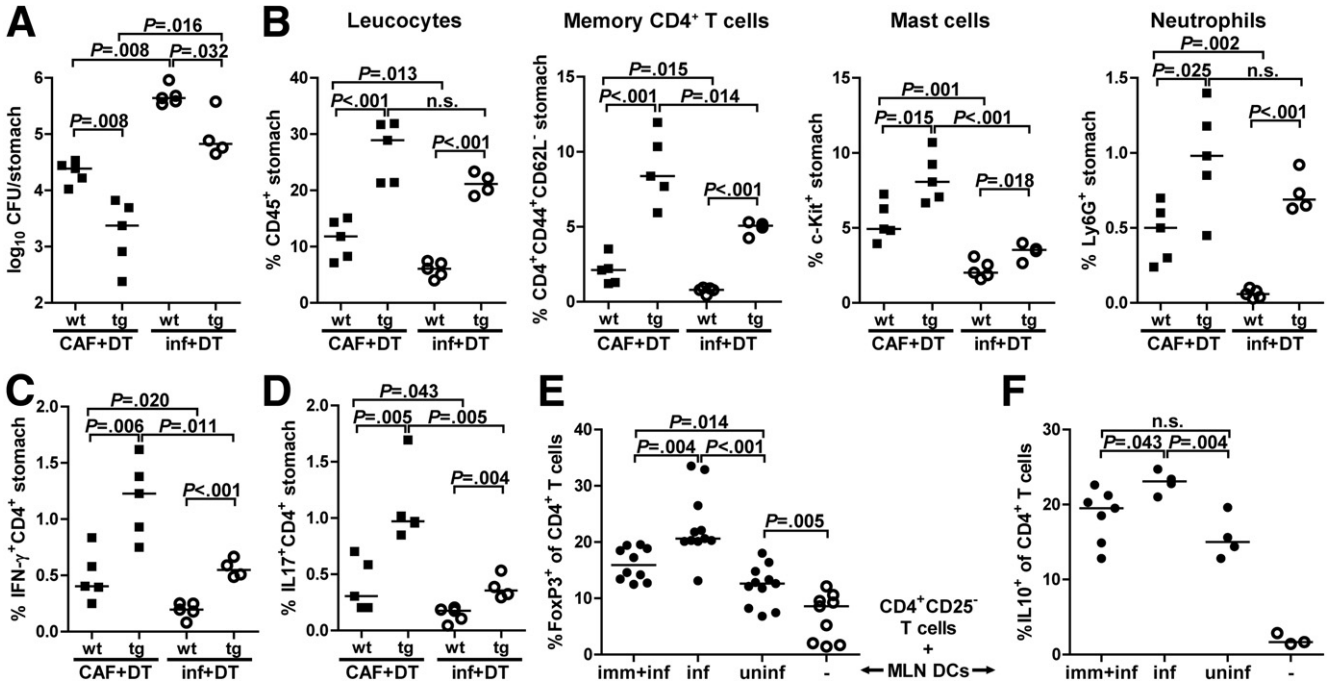


Figure 6. Treg depletion during challenge infection improves vaccine-induced protection; DCs from *H pylori*–infected mice induce Treg ex vivo. (*A*) Colonization levels of *FoxP3*-EGFP-DTR tg and wild-type mice vaccinated SC with CAF01, relative to infected controls; all mice received DT. (*B–D*) Gastric infiltration in percent of total stomach cells of leukocytes ($CD45^+$), activated memory $CD4^+$ T cells ($CD44^+CD62L^-$), mast cells (c-Kit^+), and neutrophils (Ly6-G^+; *B*) as well as IFN-γ– and IL-17–producing $CD4^+$ T cells (*C–D*) for the mice shown in *panel A*. Proportions of $FoxP3^+$ (*E*), and IL-10^+ (*F*) $CD4^+$ T cells converted from naïve $CD4^+CD25^-$ T cells upon coculture with MLN-derived DCs from immunized, infected, or uninfected mice or cultured without DCs. Pooled data from 3 independent experiments are shown in *panel E*.

challenge phase by regular IP administration of DT. Under conditions of DT administration in 2-day intervals, a frequency that was well-tolerated by the animals, the efficiency of DC depletion at all study end points was ~50% in the stomach, MLN, and spleen (Supplementary Figure 4 and data not shown). Interestingly, mice with such reduced DC populations were able to control the infection significantly better than untreated mice or nontransgenic DT-treated littermates, a phenomenon that was observed consistently in both the CT- and CAF01-immunized treatment arms (Figure 7*A*). In accordance with this finding, increased amounts of $CD45^+$ leukocytes, memory T cells, neutrophils, and mast cells were detected in gastric mucosal single-cell preparations of immunized mice with reduced DC populations compared to nondepleted immunized controls (Figure 7*B*). Generally stronger immune infiltration into the gastric mucosa was also observed histologically (Figure 7*C*); DT-depleted immunized mice further exhibited stronger IFN-γ and IL-17 responses in the gastric mucosa and in the MLN than nondepleted immunized mice as shown by real-time reverse transcription polymerase chain reaction or intracellular cytokine staining (Figure 7*D–E* and Supplementary Figure 5). The partial depletion of DCs during infection of nonimmunized animals did not reduce their bacterial burden or affect any of the other assessed infection correlates (Figure 7*A–E*, and data not shown). Speculating that the relative resistance of SS1-infected naïve mice to (partial) DC depletion was due to the comparatively poor immunogenicity of SS1, we repeated the experimental infection with the parental strain of SS1, PMSS1. PMSS1 retains a fully functional Cag pathogenicity island[37] and, as a consequence, initiates stronger immune activation and causes more severe immunopathology than its less virulent, mouse-adapted derivative.[37] DC depletion during PMSS1 infection indeed resulted in lower colonization levels and higher gastric infiltration of $CD4^+$ T cells compared to nondepleted controls (Supplementary Figure 6). Taken together, these findings indicate that tolerogenic mechanisms mediated by DCs and regulatory T cells counteract host immunity to *H pylori* and need to be overcome in order to achieve sterilizing protection.

Discussion

In this study, we provide evidence for the efficacy of a novel mycobacterial cell wall–derived adjuvant CAF01 in *H pylori*–specific vaccination. CAF01 is expected to be safe and has recently been approved for clinical testing of a new mycobacterial subunit vaccine formulation. CAF01-mediated protection in the context of *H pylori* infection was clearly restricted to parenteral routes of vaccine delivery, and was further dependent on MHC class II–restricted T cells of both Th1 and Th17 subsets. Our data thus confirm and extend several previous studies reporting that protective immunity to *Helicobacter* in small rodent models does not require humoral responses,[31–33] but rather is entirely $CD4^+$ T-cell–medi-

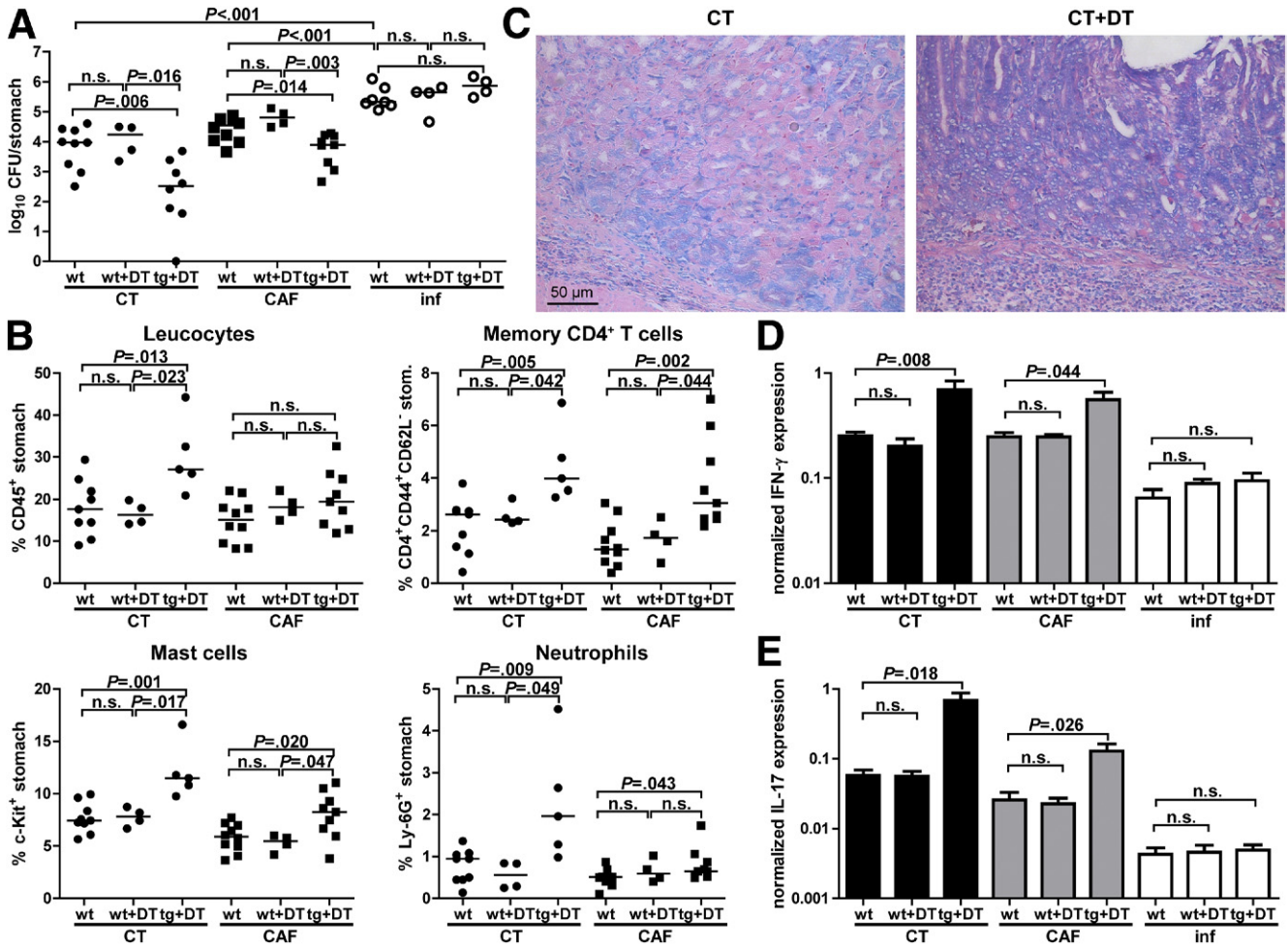


Figure 7. Vaccination outcomes improve upon DC depletion. (*A*) Colonization levels of CT-immunized, CAF01-immunized, and infected-only *CD11c*-DTR tg (tg) compared to wild-type (wt) mice, treated (+DT) or not with DT every 2 days during challenge infection. (*B*) Gastric infiltration of leukocytes ($CD45^+$), memory $CD4^+$ T cells ($CD44^+CD62L^-$), mast cells (c-Kit^+), and neutrophils (Ly6-G^+) in immunized *CD11c*-DTR tg and wild-type mice, treated (+DT) or not with DT. (*C*) Giemsa-stained histological sections of representative CT-immunized and CT-immunized/DC-depleted mice, illustrating immune cell infiltration into the gastric mucosa. (*D–E*) Real-time reverse transcription polymerase chain reaction results, normalized to glyceraldehyde-3-phosphate dehydrogenase, for (*D*) IFN-γ and (*E*) IL-17 expressions in *CD11c*-DTR tg and wild-type immunized and/or infected mice, treated (+DT) or not with DT. Pooled data from 3 independent experiments are shown.

ated[17,38] and dependent on Th1[7,15,39] and/or Th17[5,18] cytokines. By comparing immunological parameters of effective and ineffective delivery routes, we were able to identify a number of correlates that proved to be tightly associated with protection. Gastric mucosal preparations optimized to capture both lamina propria and intraepithelial immune cell infiltrates contained large numbers of mucosa-infiltrating IFN-γ– and IL-17–positive (memory) T cells, mast cells, and neutrophils, suggesting that these populations contribute to the elimination of the bacteria as proposed previously.[5,34]

Possibly the least expected finding of the present study relates to the role of DCs in protective immunity to *H pylori*. We have shown recently that the balance between *H pylori*–specific tolerance and immunity determines disease outcome in a model of gastric preneoplastic pathology induced by a particularly virulent, Cag pathogenicity island-positive *H pylori* isolate.[37] In this disease model, mice that had been infected during the neonatal tolerance window—in which the newborn immune system is immature and naturally skewed toward regulatory rather than immunogenic immune responses—were entirely protected from the immunopathological consequences of infection that are a hallmark of mice infected as adults. In this scenario, DCs are believed to act as important mediators of tolerance induction and maintenance through their ability to induce antigen-specific regulatory T cells in the periphery. In support of this notion, Kao et al recently reported that *H pylori*–infected DCs can suppress Th17 immunity and skew the immune response toward a regulatory phenotype.[40]

Mice that have developed tolerance to the infection are not only protected from gastric immunopathology but are also colonized with *H pylori* at significantly higher densities.[37] This observation prompted us to attempt to improve vaccine efficacy by overruling DC- and Treg-mediated tolerance mechanisms during the challenge phase of our immunization protocols. Indeed, even the partial depletion of all $CD11c^+$ populations that could be

achieved without the lethal side effects that are a hallmark of continuous DT treatment of *CD11c*-DTR-transgenic mice was sufficient to significantly increase the efficacy of both the CAF01- and the CT-adjuvanted vaccines. The reduced bacterial counts recovered from the DC-depleted and Treg-depleted mice were confirmed by the immunological correlates of protection outlined here. The improved protection of DC/Treg-depleted mice appeared to be due to the more efficient induction of T-cellular recall responses. In conclusion, we introduce here a novel vaccine formulation incorporating a mycobacterial cell wall glycolipid that confers Th1/Th17-mediated protective immunity to experimental *H pylori* infection. We further show that the tolerogenic functions of DCs that are activated by live *H pylori* must be overcome in order to achieve optimal protective immunity to this important human gastrointestinal pathogen.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi:10.1053/j.gastro.2011.04.009.

References

1. Marshall BJ, Windsor HM. The relation of *Helicobacter pylori* to gastric adenocarcinoma and lymphoma: pathophysiology, epidemiology, screening, clinical presentation, treatment, and prevention. Med Clin North Am 2005;89:313–344.
2. Mégraud F. *H pylori* antibiotic resistance: prevalence, importance, and advances in testing. Gut 2004;53:1374–1384.
3. Zullo A, Hassan C, Eramo A, et al. Gastric cancer following *Helicobacter pylori* eradication in duodenal ulcer patients. Digest Liver Dis 2006;38:528–529.
4. Rokkas T, Pistiolas D, Sechopoulos P, et al. The long-term impact of *Helicobacter pylori* eradication on gastric histology: a systematic review and meta-analysis. Helicobacter 2007;12(Suppl 2):32–38.
5. DeLyria ES, Redline RW, Blanchard TG. Vaccination of mice against *H pylori* induces a strong Th-17 response and immunity that is neutrophil dependent. Gastroenterology 2009;136:247–256.
6. Mueller A, O'Rourke J, Chu P, et al. Protective immunity against *Helicobacter* is characterized by a unique transcriptional signature. Proc Natl Acad Sci 2003;100:12289–12294.
7. Sayi A, Kohler E, Hitzler I, et al. The $CD4^+$ T cell-mediated IFN-gamma response to *Helicobacter* infection is essential for clearance and determines gastric cancer risk. J Immunol 2009;182:7085–7101.
8. Chen M, Lee A, Hazell S. Immunisation against gastric *Helicobacter* infection in a mouse/*Helicobacter felis* model. Lancet 1992;339:1120–1121.
9. Banerjee S, Medina-Fatimi A, Nichols R, et al. Safety and efficacy of low dose *Escherichia coli* enterotoxin adjuvant for urease based oral immunisation against *Helicobacter pylori* in healthy volunteers. Gut 2002;51:634–640.
10. Sougioultzis S, Lee CK, Alsahli M, et al. Safety and efficacy of *E. coli* enterotoxin adjuvant for urease-based rectal immunization against *Helicobacter pylori*. Vaccine 2002;21:194–201.
11. Kotloff KL, Sztein MB, Wasserman SS, et al. Safety and immunogenicity of oral inactivated whole-cell *Helicobacter pylori* vaccine with adjuvant among volunteers with or without subclinical infection. Infect Immun 2001;69:3581–3590.
12. DiPetrillo MD, Tibbetts T, Kleanthous H, et al. Safety and immunogenicity of phoP/phoQ-deleted *Salmonella typhi* expressing *Helicobacter pylori* urease in adult volunteers. Vaccine 1999;18:449–459.
13. Bumann D, Metzger WG, Mansouri E, et al. Safety and immunogenicity of live recombinant *Salmonella enterica* serovar *Typhi* Ty21a expressing urease A and B from *Helicobacter pylori* in human volunteers. Vaccine 2001;20:845–852.
14. Aebischer T, Bumann D, Epple HJ, et al. Correlation of T cell response and bacterial clearance in human volunteers challenged with *Helicobacter pylori* revealed by randomised controlled vaccination with Ty21a-based Salmonella vaccines. Gut 2008;57:1065–1072.
15. Akhiani AA, Pappo J, Kabok Z, et al. Protection against *Helicobacter pylori* infection following immunization is IL-12-dependent and mediated by Th1 cells. J Immunol 2002;169:6977–6984.
16. Akhiani AA, Schon K, Lycke N. Vaccine-induced immunity against *Helicobacter pylori* infection is impaired in IL-18-deficient mice. J Immunol 2004;173:3348–3356.
17. Ermak TH, Giannasca PJ, Nichols R, et al. Immunization of mice with urease vaccine affords protection against *Helicobacter pylori* infection in the absence of antibodies and is mediated by MHC class II-restricted responses. J Exp Med 1998;188:2277–2288.
18. Velin D, Favre L, Bernasconi E, et al. Interleukin-17 is a critical mediator of vaccine-induced reduction of *Helicobacter* infection in the mouse model. Gastroenterology 2009;136:2237–2246.e1.
19. Rossi G, Ruggiero P, Peppoloni S, et al. Therapeutic vaccination against *Helicobacter pylori* in the beagle dog experimental model: safety, immunogenicity, and efficacy. Infect Immun 2004;72:3252–3259.
20. Gottwein JM, Blanchard TG, Targoni OS, et al. Protective anti-*Helicobacter* immunity is induced with aluminum hydroxide or complete Freund's adjuvant by systemic immunization. J Infect Dis 2001;184:308–314.
21. Eisenberg JC, Czinn SJ, Garhart CA, et al. Protective efficacy of anti–*Helicobacter pylori* immunity following systemic immunization of neonatal mice. Infect Immun 2003;71:1820–1827.
22. Malfertheiner P, Schultze V, Rosenkranz B, et al. Safety and immunogenicity of an intramuscular *Helicobacter pylori* vaccine in noninfected volunteers: a phase I study. Gastroenterology 2008;135:787–795.
23. Werninghaus K, Babiak A, Gross O, et al. Adjuvanticity of a synthetic cord factor analogue for subunit *Mycobacterium tuberculosis* vaccination requires FcRgamma-Syk-Card9-dependent innate immune activation. J Exp Med 2009;206:89–97.
24. Korsholm KS, Agger EM, Foged C, et al. The adjuvant mechanism of cationic dimethyldioctadecylammonium liposomes. Immunology 2007;121:216–226.
25. Kamath AT, Rochat A-F, Christensen D, et al. A liposome-based mycobacterial vaccine induces potent adult and neonatal multifunctional T cells through the exquisite targeting of dendritic cells. PLoS ONE 2009;4:e5771.
26. Davidsen J, Rosenkrands I, Christensen D, et al. Characterization of cationic liposomes based on dimethyldioctadecylammonium and synthetic cord factor from *M. tuberculosis* (trehalose 6,6′-dibehenate)—a novel adjuvant inducing both strong CMI and antibody responses. Biochim Biophys Acta Biomembr 2005;1718:22–31.
27. Agger EM, Rosenkrands I, Hansen J, et al. Cationic liposomes formulated with synthetic mycobacterial cordfactor (CAF01): a versatile adjuvant for vaccines with different immunological requirements. PLoS ONE 2008;3:e3116.
28. Gu H, Zou YR, Rajewsky K. Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. Cell 1993;73:1155–1164.
29. Lahl K, Loddenkemper C, Drouin C, et al. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. J Exp Med 2007;204:57–63.

30. Lee A, O'Rourke J, Ungria MCD, et al. A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. Gastroenterology 1997;112:1386–1397.
31. Blanchard TG, Czinn SJ, Redline RW, et al. Antibody-independent protective mucosal immunity to gastric *Helicobacter* infection in mice. Cellular Immunology 1999;191:74–80.
32. Garhart CA, Nedrud JG, Heinzel FP, et al. Vaccine-induced protection against *Helicobacter pylori* in mice lacking both antibodies and interleukin-4. Infect Immun 2003;71:3628–3633.
33. Sutton P, Wilson J, Kosaka T, et al. Therapeutic immunization against *Helicobacter pylori* infection in the absence of antibodies. Immunol Cell Biol 2000;78:28–30.
34. Velin D, Bachmann D, Bouzourene H, et al. Mast cells are critical mediators of vaccine-induced *Helicobacter* clearance in the mouse model. Gastroenterology 2005;129:142–155.
35. Toller IM, Hitzler I, Sayi A, et al. Prostaglandin E2 prevents *Helicobacter*-induced gastric preneoplasia and facilitates persistent infection in a mouse model. Gastroenterology 2010;138:1455–1467, 1467.e1-4.
36. Mueller A, Sayi A, Hitzler I. Protective and pathogenic functions of T cells are inseparable during the *Helicobacter*-host interaction. Discov Med 2009;8:68–73.
37. Arnold I, Lee JY, Amieva MR, et al. Tolerance rather than immunity protects from *Helicobacter pylori*–induced gastric preneoplasia. Gastroenterology 2011;140:199–209.
38. Pappo J, Torrey D, Castriotta L, et al. *Helicobacter pylori* infection in immunized mice lacking major histocompatibility complex class I and class II functions. Infect Immun. 1999;67:337–341.
39. Taylor JM, Ziman ME, Canfield DR, et al. Effects of a Th1- versus a Th2-biased immune response in protection against *Helicobacter pylori* challenge in mice. Microb Pathog 2007;44:20–27.
40. Kao JY, Zhang M, Miller MJ, et al. *Helicobacter pylori* immune escape is mediated by dendritic cell-induced Treg skewing and Th17 suppression in mice. Gastroenterology 2010;138:1046–1054.

Received August 18, 2010. Accepted April 8, 2011.

Reprint requests

Address requests for reprints to: Anne Müller, PhD, Institute of Molecular Cancer Research, University of Zürich, Winterthurerstr. 190, 8057 Zürich, Switzerland. e-mail: mueller@imcr.unizh.ch; fax: +41 44 635 3484.

Acknowledgments

The authors would like to thank Tim Sparwasser and Regeneron Pharmaceuticals for providing *foxP3*-EGFP-DTR transgenic and interleukin-23p19$^{-/-}$ mice, respectively. We are grateful to Maries van den Broek, Roberto Speck, and Josef Jiricny for critical discussions. Isabelle Arnold, Isabella Toller, Esther Kohler, Vanessa Craig, and Ayca Sayi are thanked for experimental assistance and discussions.

Conflicts of interest

The authors disclose no conflicts.

Funding

This study was funded by grants from the Cancer League of the Canton of Zurich and the Swiss National Science Foundation (to AM). Additional funding was supplied by the University Research Priority Program in Systems Biology.

Supplementary Methods

Enzyme-Linked Immunosorbent Assay and Real-Time Reverse Transcription Polymerase Chain Reaction

For determination of *H pylori*–specific antibody titers, 96-well enzyme-linked immunosorbent assay plates were coated over night with 10 μg/well of *H pylori* sonicate in carbonate buffer and incubated with 1:10.000 diluted sera or 5 μg mucosal protein extracts; bound antibodies were detected with horseradish peroxidase–coupled goat anti-mouse IgG, IgG1, or IgG2c (all AbD Serotec, Kidlington, UK). IFN-γ secreted by cultured MLN and stomach cells was quantified by enzyme-linked immunosorbent assay (R&D Systems). For real-time RT-PCR, total RNA was isolated from one-sixth of every stomach (antrum and corpus) using NucleoSpin RNA II kits (Macherey-Nagel, Düren, Germany). The corresponding complementary DNA served as a template for real-time polymerase chain reaction performed using the LightCycler 480 SYBR Green I master kit (Roche, Basel, Switzerland). Absolute values of IFN-γ and IL-17 expression were normalized to GAPDH expression (conditions: Tm 55°C, 50 cycles; primers: glyceraldehyde-3-phosphate dehydrogenase fw GAC ATT GTT GCC ATC AAC GAC C / glyceraldehyde-3-phosphate dehydrogenase rv CCC GTT GAT GAC CAG CTT CC, IFN-γ fw CAT GGC TGT TTC TGG CTG TTA CTG / IFN-γ rv GTT GCT GAT GGC CTG ATT GTC TTT, IL-17 fw GCT CCA GAA GGC CCT CAG A / IL-17 rv AGC TTT CCC TCC GCA TTG A).

Quantification of H pylori *Colonization by Colony Count Assay*

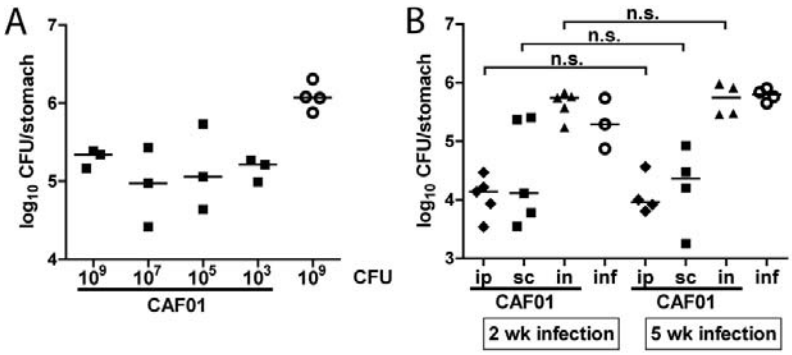
For the quantitative assessment of *H pylori* colonization, one section of each stomach was transferred to a tube containing Brucella broth and homogenized with an Ultra Turrax homogenizer (John Morris Scientific Ltd, Chatswood, Australia). Serial dilutions were plated on horse blood plates to determine bacterial loads.

Treg Conversion Assay

DCs were isolated from MLN single cell suspensions using mouse-specific CD11c microbeads (Miltenyi Biotec). $CD4^+CD25^-$ T cells were prepared from single-cell suspensions of uninfected C57BL/6 spleens by immunomagnetic sorting (R&D Systems). DCs and T cells were cocultured at a ratio of 1:2 (50,000 DCs to 100,000 T cells) in RPMI containing 10% fetal calf serum, 10 ng/mL recombinant transforming growth factor–β (PeproTech, Rocky Hill, NJ), 10 ng/mL recombinant IL-2 (R&D Systems), and 1 μg/mL anti-CD3ε (BD Bioscience). After 72 hours of coculture, the cells were stained first for CD4 and then, after fixation, for FoxP3. The percentage of $FoxP3^+$ $CD4^+$ T cells was assessed by flow cytometric analysis.

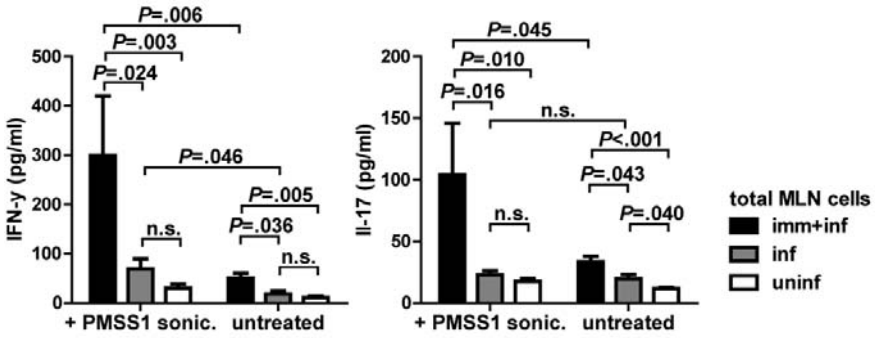
Supplementary Figures

Supplementary Figure 1



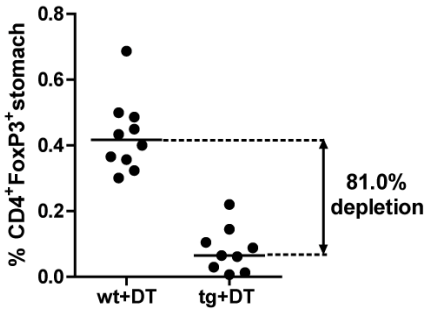
Suppl. Figure 1. The outcome of *H. pylori* challenge infection does not depend on the challenge dose and remains the same at later study endpoints. (*A*), Colonization levels of CAF01 s.c.-immunized mice two weeks after challenge with *H. pylori* doses ranging from 10^3 to 10^9 bacteria and unimmunized controls infected with 10^9 bacteria. (*B*), Colonization levels of mice immunized with CAF01 i.p., s.c. or i.n. as well as unimmunized controls as determined two or five weeks after challenge infection, respectively.

Supplementary Figure 2



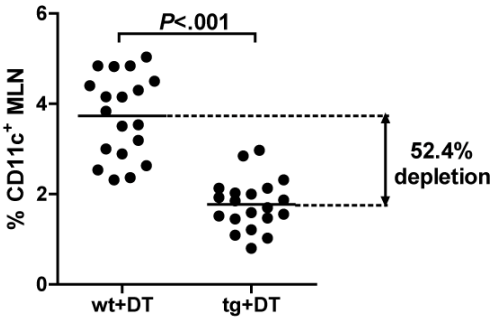
Suppl. Figure 2. *Helicobacter*-specific response elicited *ex vivo* in MLN cells of mice immunized against and infected with *H. pylori* strain PMSS1. Total MLN cells were isolated from mice immunized with CAF01 and PMSS1 sonicate and subsequently challenged with PMSS1, as well as infected and uninfected controls. IFN-γ and IL-17 secretion of unstimulated cells and cells restimulated with 10µg/ml PMSS1 sonicate was determined by ELISA after 3 days.

Supplementary Figure 3



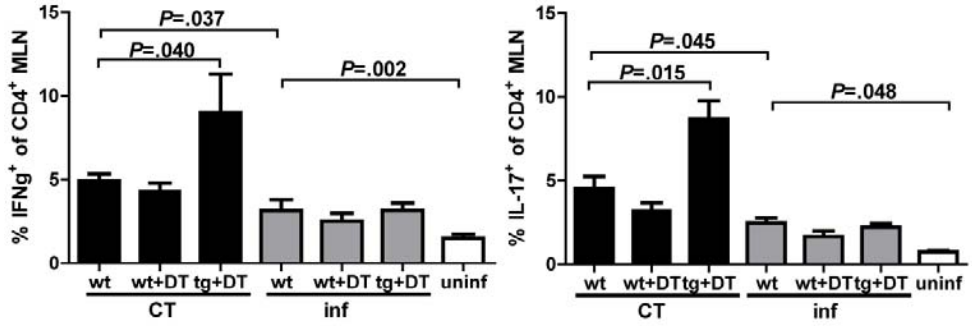
Suppl. Figure 3. Efficiency of Treg depletion. Percentage of $CD4^+FoxP3^+$ cells in the stomach cell preparations of DT-treated wild type (wt) and *FoxP3*-EGFP-DTR tg (tg) mice.

Supplementary Figure 4



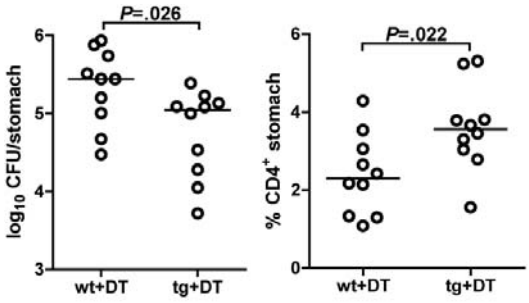
Suppl. Figure 4. Efficiency of DC depletion. Percentage of $CD11c^+$ cells in the MLN of DT-treated wild type (wt+DT) and *CD11c*-DTR tg (tg+DT) mice.

Supplementary Figure 5



Suppl. Figure 5. DC depletion during infection increases IFN-γ- and IL-17- producing CD4$^+$ T cell populations in the MLN of vaccinated mice. Proportion of IFN-γ$^+$ and IL-17$^+$ of total CD4$^+$ T cells in MLN of CT-immunized/infected or only infected wild type (wt) and *CD11c*-DTR tg (tg) mice, respectively, DT-treated (+DT) or not, as well as uninfected controls.

Supplementary Figure 6



Suppl. Figure 6. Depletion of dendritic cells reduces the bacterial burden of PMSS1-infected mice. Colonization levels and gastric CD4$^+$ T cell infiltration of wild type (wt+DT) and *CD11c*-DTR tg (tg+DT) mice infected with PMSS1 and DT-treated during two weeks of infection.

3.4 The C-terminally encoded, MHC class II-restricted T-cell antigenicity of the *Helicobacter pylori* virulence factor CagA promotes gastric preneoplasia

article published in Journal of Immunology, 2011

authors: Isabelle Arnold, Iris Hitzler, Daniela Engler, Mathias Oertli, Else-Marie Agger and Anne Müller

contributions: I performed and analyzed parts of the vaccination and tolerance studies and helped with the revision. IA and AM designed the study, IA conducted research with help by DE and MO, AM wrote the manuscript. EMA provided reagents.

Published April 25, 2011, doi:10.4049/jimmunol.1003472

The C-Terminally Encoded, MHC Class II-Restricted T Cell Antigenicity of the *Helicobacter pylori* Virulence Factor CagA Promotes Gastric Preneoplasia

Isabelle C. Arnold,* Iris Hitzler,* Daniela Engler,* Mathias Oertli,* Else Marie Agger,† and Anne Müller*

Chronic infection with the human bacterial pathogen *Helicobacter pylori* causes gastritis and predisposes carriers to an increased gastric cancer risk. Consequently, *H. pylori*-specific vaccination is widely viewed as a promising strategy of gastric cancer prevention. *H. pylori* strains harboring the Cag pathogenicity island (PAI) are associated with particularly unfavorable disease outcomes in humans and experimental rodent models. We show in this study using a C57BL/6 mouse model of Cag-PAI$^+$ *H. pylori* infection that the only known protein substrate of the Cag-PAI–encoded type IV secretion system, the cytotoxin-associated gene A (CagA) protein, harbors MHC class II-restricted T cell epitopes. Several distinct nonoverlapping epitopes in CagA's central and C-terminal regions were predicted in silico and could be confirmed experimentally. CagA$^+$ infection elicits CD4$^+$ T cell responses in mice, which are strongly enhanced by prior mucosal or parenteral vaccination with recombinant CagA. The adoptive transfer of CagA-specific T cells to T cell-deficient, *H. pylori*-infected recipients is sufficient to induce the full range of preneoplastic immunopathology. Similarly, immunization with a cholera toxin-adjuvanted, CagA$^+$ whole-cell sonicate vaccine sensitizes mice to, rather than protects them from, *H. pylori*-associated gastric cancer precursor lesions. In contrast, *H. pylori*-specific tolerization by neonatal administration of *H. pylori* sonicate in conjunction with a CD40L-neutralizing Ab prevents *H. pylori*-specific, pathogenic T cell responses and gastric immunopathology. We conclude that active tolerization may be superior to vaccination strategies in gastric cancer prevention. ***The Journal of Immunology*****, 2011, 186: 000–000.**

Persistent gastric infection with the bacterial pathogen *Helicobacter pylori* results in chronic gastritis (1) and predisposes carriers to a high risk for development of gastric and duodenal ulcers, gastric cancer, and gastric MALT lymphoma (2–4). Despite a recent decline in infection rates in industrialized countries, the prevalence rate of *H. pylori* remains at nearly 100% in the developing world (5). The infection can be eradicated in a majority of individuals by antibiotic therapy; however, resistance rates are rising (6), and vaccination against *H. pylori* is viewed as a cost-effective alternative to eradication therapy (7). Several vaccination regimens induce protective immunity in animal models; these include subunit vaccines containing *H. pylori* urease (8), neutrophil-activating protein (9), or adhesin A (10), but also recombinant live *Salmonella* vaccines expressing *Helicobacter* Ags (11). Clinical trials have demonstrated the immunogenicity of experimental vaccines in human volunteers (12–14). However, all current vaccine development efforts are hampered by their failure to achieve sterilizing immunity in rodent models, let alone in humans.

Helicobacter strains harboring the cytotoxin-associated gene A-encoded virulence factor CagA have been associated with high levels of gastric inflammation (15) and an increased gastric cancer risk compared with CagA$^-$ strains (16, 17). CagA is the only known protein substrate of a pathogenicity island-encoded type IV secretion system (T4SS), which allows the bacteria to deliver the virulence factor directly into their host cell's cytosol (18). Upon injection, CagA is tyrosine phosphorylated on C-terminal motifs, leading to the loss of cell-to-cell contacts, cell scattering, and increased motility (19). CagA delivery to the host cell further disrupts cell polarity (20) and allows the bacteria to colonize the apical surface of cultured cells (21). In vivo, transgenic expression of CagA under a stomach-specific promoter is by itself sufficient to induce epithelial hyperplasia and, in a subset of mice, gastric polyps and adenocarcinoma (22), implying that CagA can function as a bacterial oncoprotein. In a Mongolian gerbil model of CagA$^+$ *H. pylori* infection, the bacteria induce gastric cancer precursor lesions that resemble *H. pylori*-associated lesions in humans (23). We have recently introduced a C57BL/6 mouse model of infection with a Cag$^+$ *H. pylori* patient isolate that induces atrophic gastritis, epithelial hyperplasia, and intestinal metaplasia in its host in a T4SS-dependent manner (24). In this model, the age of the host at the time of infection determines disease outcome. Mice that are experimentally infected during the neonatal period develop immunological tolerance rather than immunity to *H. pylori* and are protected from the immunopathological T cell responses that are a hallmark of CagA$^+$ infection in adults (24).

*Institute of Molecular Cancer Research, University of Zurich, 8057 Zurich, Switzerland; and †Department of Infectious Disease Immunology, Statens Serum Institute, 2300 Copenhagen, Denmark

Received for publication October 20, 2010. Accepted for publication March 30, 2011.

This work was supported by grants from the Swiss National Science Foundation (to A.M.), the Swiss Cancer League (to A.M.), the Cancer League of the Canton of Zurich (to A.M.), and the University Research Priority Program in Systems Biology/Functional Genomics.

Address correspondence and reprint requests to Prof. Anne Müller, Institute of Molecular Cancer Research, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland. E-mail address: mueller@imcr.uzh.ch

The online version of this article contains supplemental material.

Abbreviations used in this article: CagA, cytotoxin-associated gene A; CT, cholera toxin; DC, dendritic cell; MLN, mesenteric lymph node; PAI, pathogenicity island; p.i., postinfection; PMSS1, premouse Sydney strain 1; rCagA, recombinant CagA; T4SS, type IV secretion system.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1003472

In this study, we pursued the hypothesis that the CagA protein itself functions as a "pathogenic," procarcinogenic T cell Ag. By analyzing the antigenicity of recombinantly expressed full-length CagA, as well as truncated fragments and in silico-predicted CagA peptides, we have identified several distinct MHC class II-restricted T cell epitopes in the central and C-terminal regions of the protein. We further show in this article that immunization with CagA, either systemically or mucosally, greatly enhances the host's T cell response to challenge infection but fails to afford protective immunity. Immunization with a CagA$^+$ whole-cell sonicate vaccine sensitizes mice to, rather than protects them from, gastric cancer precursor lesions. In contrast, active tolerization of neonatal mice by administration of *H. pylori* sonicate in conjunction with a CD40L blocking Ab before experimental infection prevents pathogenic T cell responses and protects mice from preneoplastic gastric changes. We conclude that tolerization strategies may hold more promise than vaccination for the prevention and management of *H. pylori*-associated gastric disease manifestations.

Materials and Methods

Animal experimentation and H. pylori *cultures*

C57BL/6 and TCR-$\beta^{-/-}$ BL6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and bred at a University of Zurich specific pathogen-free facility. Mice were maintained in individually ventilated cages and included in studies at 6 wk of age. All animal experimentation was conducted in accordance with cantonal and federal guidelines for the care and use of laboratory animals, and was reviewed and approved by the Zurich cantonal veterinary office (permit number 63/2008 to A.M.). Mice were immunized four times at weekly intervals with 100 μg recombinant CagA (rCagA) or 1 mg *H. pylori* (premouse Sydney strain 1 [PMSS1]) sonicate, either adjuvanted with 10 μg cholera toxin (CT; List Biologicals, Campbell, CA) and given orally, or administered s.c. with CAF01. A stable CAF01 formulation consisting of dimethyldioctadecylammonium bromide and a,a′-trehalose 6,6′-dibehenate (Avanti Polar Lipids) was prepared by the lipid film hydration method as previously described (25). All mice were challenged 1 wk after the last immunization with *H. pylori* PMSS1 by oral gavage of 10^8 bacteria. Anti-CD40L mAb (clone MR1) and anti-CD40 mAb (clone FGK 4.5; both Bio X Cell, West Lebanon, PA) were i.p. injected at 50 or 75 μg/dose into neonatal mice on days 7, 10, 12, and 14 after birth. Neonatal mice were infected at 7 d of age with 2×10^7 CFU *H. pylori* PMSS1 in 50 μl. For adoptive transfer experiments, 300,000 immunomagnetically sorted splenic CD4$^+$CD25$^-$ T cells (CD4$^+$CD25$^+$ T cell purification kit; R&D Systems, Minneapolis, MN) were injected into the tail veins of TCR-$\beta^{-/-}$ mice. The *H. pylori* PMSS1 used in this study, as well as agar and liquid culture conditions, was described in detail previously (24).

Preparation of gastric tissue and gastric/mesenteric lymph node single-cell suspensions: assessment of H. pylori *colonization, histopathology, and IFN-γ production*

Stomachs were retrieved and dissected longitudinally into equally sized pieces. For quantitative assessment of *H. pylori* colonization, one section of each stomach was homogenized in *Brucella* broth, and serial dilutions were plated on horse blood plates for colony counting. For the quantitative assessment of gastric histopathology, Giemsa-stained, paraffin-embedded, longitudinal stomach sections ranging from the forestomach/corpus junction all the way to the duodenum were scored independently and in a blinded fashion by two experimenters. Scores on a scale of 0–6 were assigned for the parameters' chronic inflammation (in the antrum and corpus areas), as well as corpus atrophy, epithelial hyperplasia, and metaplasia. For the characterization of gastric immune cell infiltrates, one sixth of every stomach (antrum and corpus) was digested in 1 mg/ml collagenase (Sigma-Aldrich), disrupted between glass slides, filtered through a cell strainer (40 μm; BD Biosciences, San Jose, CA), and processed for flow cytometry (see later). Mesenteric lymph nodes (MLN) of individual mice were disrupted by collagenase digestion followed by repeated pipetting and filtering; single-cell suspensions were either subjected to immunomagnetic cell isolation (CD4$^+$CD25$^+$ T cell purification kit; R&D Systems) or cultured for 4 d in round-bottom 96-well plates for assessment of IFN-γ secretion by ELISA (BD Biosciences) or by intracellular cytokine staining (see later).

Flow cytometry

The following Abs were used for FACS analysis: IFN-γ–PE–Cy7, CD4-FITC (BD Biosciences, San Jose, CA), CD45-PB, CD62L-allophycocyanin, CD44-PB, GR-1 Ly6-G–allophycocyanin, and rat anti-mouse c-Kit/CD117 (all from BioLegend, San Diego, CA). For intracellular IFN-γ staining, cells were restimulated and blocked for 5 h in medium containing 2.5 μg/ml brefeldin A (AppliChem, Darmstadt, Germany), 0.2 μM ionomycin (Santa Cruz Biotechnology, Santa Cruz, CA), and 50 ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich). Cells were first stained for CD4 and then fixed in 4% paraformaldehyde and stained for IFN-γ in 10% saponin permeabilization buffer. Flow cytometric analysis was performed on a CyanADP instrument and analyzed with Summit software (Beckman Coulter, Brea, CA).

Generation of rCagA fragments and synthetic peptides, and assessment of CagA-specific T cell responses

For the generation of full-length rCagA and its fragments, PCR products were obtained with HotStar High Fidelity DNA Polymerase (Qiagen) and cloned into the BamH1 and SalI restriction sites of the pGEX 4T3 expression vector with an N-terminal GST-tag. All primer sequences and PCR conditions are listed in Supplemental Table I. Protein expression was induced in *Escherichia coli* BL21 by isopropyl β-D-thiogalactopyranoside for 4 h at 27°C, followed by glutathione Sepharose affinity chromatography. MHC class II-binding 15-aa peptides were predicted with Rankpep (http://immunax.dfci.harvard.edu/Tools/rankpep.html) and synthesized by Thermo Fisher Scientific (peptide 11: NFNKAVAEAKNTGNY; peptide 14: EEPIYAQVAKKVNAK; peptide 15: AESAKKVPASLSAKL; peptide 16: TGYYCLAEENAEHGI). For the analysis of CagA-specific T cell responses, dendritic cells (DC) were enriched from MLN suspensions of TCRβ$^{-/-}$ mice by CD11c-specific immunomagnetic isolation (BD Biosciences). A total of 80,000 DC were pulsed overnight with 50 μg/ml rCagA or its fragments and cocultured with 150,000 immunomagnetically isolated MLN-derived CD4$^+$CD25$^-$ T cells. CagA peptides were added directly to the cocultures at 10 μg/ml. IFN-γ production was

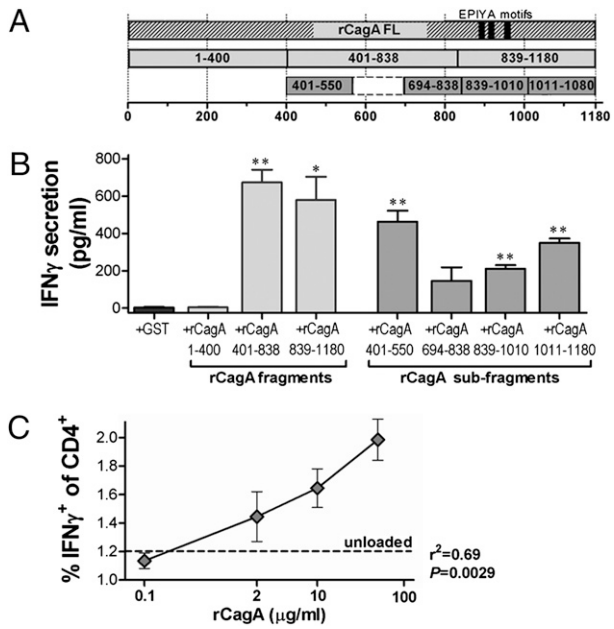


FIGURE 1. CagA harbors central and C-terminally encoded MHC class II-restricted T cell epitopes. *A*, Schematic representation of full-length CagA and the fragments used in *B*. *B* and *C*, A total of 80,000 MLN DC were pulsed with 50 μg/ml of either the GST-tag alone or the indicated CagA fragments (*B*), or with increasing concentrations of full-length rCagA (*C*), and cocultured for 4 d with 150,000 pooled CD4$^+$CD25$^-$ MLN T cells from 5 *H. pylori*-infected mice. IFN-γ secretion of cocultures was quantified by ELISA (*B*) or by intracellular cytokine staining (*C*). $*p < 0.05$, $**p < 0.01$ in relation to the GST-loaded DC. The correlation in *C* between the CagA concentration used for DC loading and the fraction of IFN-γ$^+$ cells of the total CD4$^+$ population was calculated by regression analysis; the *p* and r^2 values are indicated. Data are representative of three to five independent experiments.

assessed by ELISA (BD Biosciences) or by intracellular staining after 4 d of culture.

CagA ELISAs and IFN-γ real-time RT-PCR

For the evaluation of CagA-specific Ab titers, serum was diluted 1/10,000 and assessed in triplicate by ELISA on 96-well plates (Nunc, Roskilde, Denmark) precoated with 5 μg rCagA in carbonate buffer. Bound Abs were detected by HRP-coupled goat anti-mouse IgG, IgG1, or IgG2c (all from AbD Serotec, Kidlington, U.K.) Abs according to the manufacturer's recommendations. After addition of tetramethylbenzidine substrate (Sigma-Aldrich), the OD was measured at 655 nm. For real-time RT-PCR of IFN-γ, RNA was isolated from scraped gastric mucosa (antrum and corpus) using a NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany). A total of 1.5 μg total RNA was used for cDNA synthesis with Superscript Reverse Transcriptase III (Life Technologies). The resulting cDNA served as a template for real-time PCR performed with a LightCycler 480 using the SYBR green I master kit (Roche, Basel, Switzerland). Absolute values of IFN-γ expression were normalized to GAPDH expression. Primers and conditions are listed in Supplemental Table I.

Statistics

GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA) was used for statistical analyses. The significance of differences in histopathology categories was calculated by Mann–Whitney *U* test; all other indicated *p* values were calculated by Student *t* test. Linear regression analysis was performed when increasing concentrations of Ag were used in T cell priming experiments. In all scatter plot graphs, the medians are indicated by horizontal bars. In column bar graphs, standard deviations are indicated by vertical bars.

Results

H. pylori *CagA harbors MHC class II-restricted T cell epitopes in its central and C-terminal domains*

H. pylori harboring a functional T4SS triggers Th1 cell infiltration into the chronically infected gastric mucosa and the subsequent formation of atrophic, hyperplastic, and metaplastic lesions (24). We hypothesized that the only known T4SS substrate and strong humoral Ag, the CagA protein, might itself function as an MHC class II-restricted T cell Ag and trigger T cellular IFN-γ production. To test this possibility, we expressed and purified full-length CagA derived from *H. pylori* PMSS1, as well as fragments corresponding to the N-terminal, C-terminal, and central domains of the protein as N-terminally tagged GST fusions (Fig. 1*A*). Immunomagnetically isolated DC were pulsed with purified protein and cocultured with $CD4^+CD25^-$ T cells isolated from the gut-draining MLN of *H. pylori*-infected mice before the quantification of IFN-γ secretion. Full-length CagA, as well as the fragments corresponding to aa 400–838 and aa 838–1180, efficiently stimulated IFN-γ production of the cocultures; a fragment containing aa 1–400 was inactive in this regard, as was the GST tag itself (Fig. 1*B*, Supplemental Fig. 1). Further subcloning and testing of 150-aa-long subfragments revealed the existence of at least three to four distinct central and C-terminal T cell epitopes (Fig. 1*A*, 1*B*). T cells from infected mice generally responded more strongly than T cells from uninfected mice in this experimental setup (Supplemental Fig. 1). CagA did not trigger T cellular IFN-γ production in the absence of APCs (data not shown). IFN-γ production in the cultures was restricted to $CD4^+$ T cells as determined by intracellular staining, and was positively correlated with the CagA concentration used for DC loading, as determined by linear regression analysis (Fig. 1*C*). The combined results imply that the central and C-terminal domains of the CagA protein contain MHC class II-restricted T cell epitopes that stimulate $CD4^+$ T cells to produce and secrete IFN-γ.

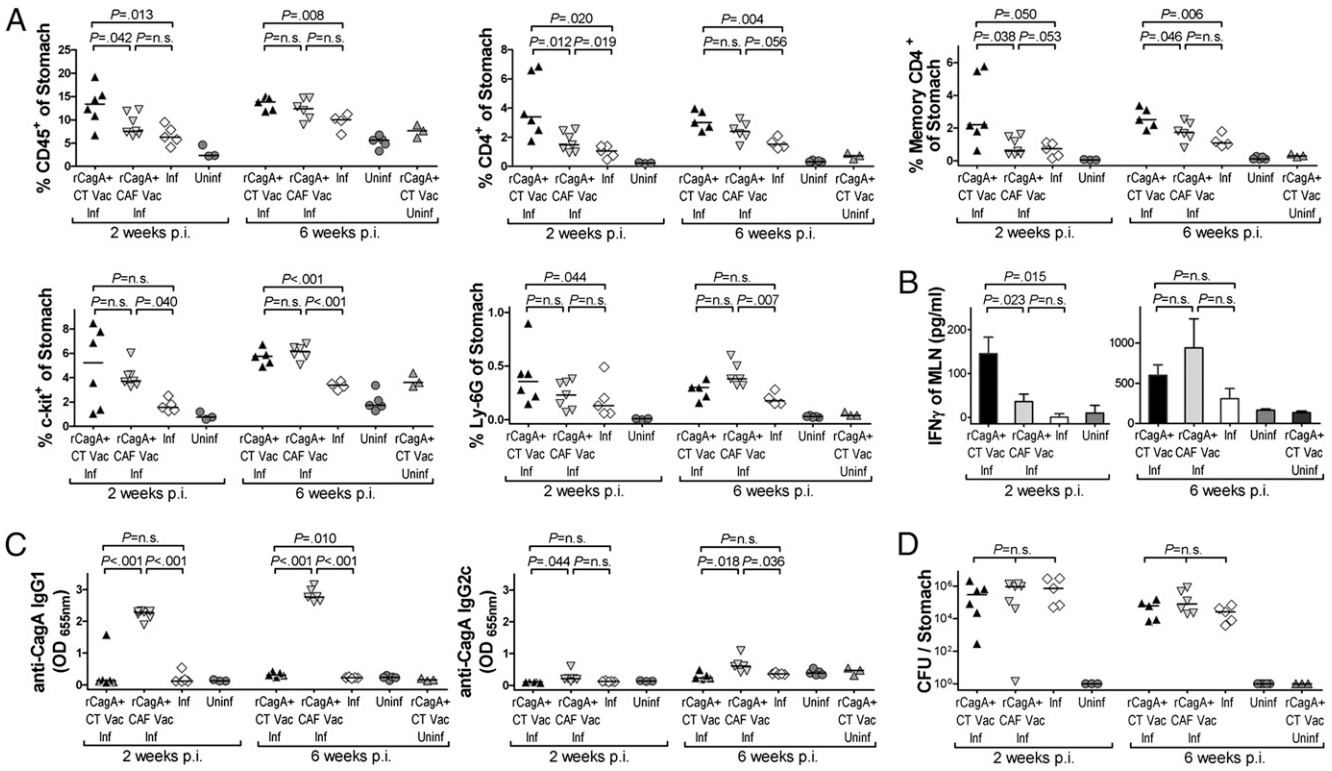


FIGURE 2. Immunization with rCagA induces local and systemic immune responses. Mice were immunized four times in weekly intervals with rCagA adjuvanted with either CT (rCagA+CT Vac) or CAF01 (rCagA+CAF Vac), challenged with live *H. pylori* together with a nonimmunized group (Inf), and sacrificed 2 and 6 wk p.i. Uninfected and immunized-only mice (rCagA+CT Vac Uninf) served as controls. *A*, Gastric infiltration of leukocytes ($CD45^+$), T cells ($CD4^+$), memory T cells ($CD44^+CD62L^-$), mast cells (c-Kit^+), and neutrophils (Ly6-G) as quantified by FACS of gastric single-cell preparations. *B*, IFN-γ secretion of MLN cultures as determined by ELISA. Averages ± SDs are shown for MLN cultures isolated from all four to seven individual mice per group. *C*, CagA-specific IgG1 and IgG2c serum titers as determined by ELISA. *D*, *H. pylori* colonization densities as determined by plating and colony counting. Data are representative of three independent immunization experiments. n.s., not significant.

Mucosal or systemic immunization with rCagA induces strong Th1-polarized responses to challenge infection but fails to confer protective immunity

To assess the immunogenicity of full-length rCagA in vivo in mucosal and systemic immunization models, we either administered CagA orally with CT or s.c. with a cationic adjuvant formulation derived from *Mycobacterium tuberculosis* (CAF01). CAF01 consists of the synthetic analog of a mycobacterial cell wall glycolipid (trehalose 6,6′-dibehenate) delivered in dimethyldioctadecylammonium liposomes; it triggers mixed Th1/Th17 responses and is successfully used in mycobacterial vaccine formulations (26). Both vaccines were administered four times at weekly intervals before challenge infection with CagA$^+$ *H. pylori*. Challenged mice were compared at 2 and 6 wk postinfection (p.i.) with nonimmunized, infected, and uninfected controls with respect to gastric infiltration of various immune cell types, T cell responses in the MLN, serum titers to CagA, and colony counts.

All immunized mice, independent of the route and adjuvant used for their vaccination, differed strongly from nonimmunized mice with respect to all parameters analyzed at both time points p.i. (Fig. 2*A–C*). CagA-vaccinated mice showed higher gastric infiltration of CD45$^+$ leukocytes, CD4$^+$ T cells, CD44$^+$CD62L$^-$ memory T cells, c-Kit$^+$ mast cells, and Ly6-G$^+$ neutrophils than infected-only controls and uninfected controls (Fig. 2*A*). *Helicobacter*-specific T cell priming and Th1 differentiation in the MLN in response to challenge infection was stronger in immunized mice as determined by IFN-γ ELISA of MLN single-cell suspension cultures of individual mice (Fig. 2*B*). A group of immunized but unchallenged mice was indistinguishable from unimmunized, uninfected controls with respect to all parameters analyzed at the 6 wk p.i. time point (Fig. 2*A*, 2*B*). High CagA-specific IgG titers were measured in the systemically immunized, but not the mucosally immunized or control infected mice (Fig. 2*C*). The IgG subclass profile further suggests a bias toward IgG1 over IgG2c production (Fig. 2*C*). The results indicate that parenteral or mucosal vaccination with rCagA enhances the cellular and humoral immune responses to challenge infection, both locally at the site of infection and in the draining lymph nodes, thereby providing evidence for the in vivo immunogenicity of CagA.

Surprisingly, however, no significant differences in colonization were observed between immunized and naive mice on challenge infection (Fig. 2*D*), indicating that immunization with adjuvanted rCagA alone does not confer protective immunity. In contrast, roughly half of all immunized mice showed widespread atrophy and hyperplasia at both 2 and 6 wk p.i., a phenomenon that is only rarely seen in infected-only controls at such early time points (Supplemental Fig. 2*A*, 2*B*). In conclusion, immunization with rCagA efficiently stimulates memory T cell responses that are reactivated on challenge infection and result in increased gastric immune cell infiltration; however, these responses fail to clear or reduce the bacteria in the vaccinated hosts.

Several distinct MHC class II-restricted T cell epitopes are encoded in the central and C-terminal domains of CagA

We next aimed to identify specific MHC class II-restricted T cell epitopes in the central and C-terminal regions of CagA. Postulating that CagA-specific T cells should be particularly abundant in CagA-vaccinated mice, and should further be enriched in the draining MLN on challenge infection, we cultured single-cell MLN preparations from individual vaccinated and control mice with rCagA. rCagA, but not the GST-tag alone, stimulated IFN-γ production by MLN cells that was stronger in the vaccinated/challenged than in the infected-only mice (Fig. 3*A*), reflecting their overall stronger reactivity to *H. pylori* (Fig. 2). MLN cultures from the same groups of mice were further restimulated in a parallel experiment with various peptides corresponding to in silico-predicted MHC class II-restricted T cell epitopes located in the central and C-terminal domains of CagA (Fig. 3*B*, 3*C*). Four of the

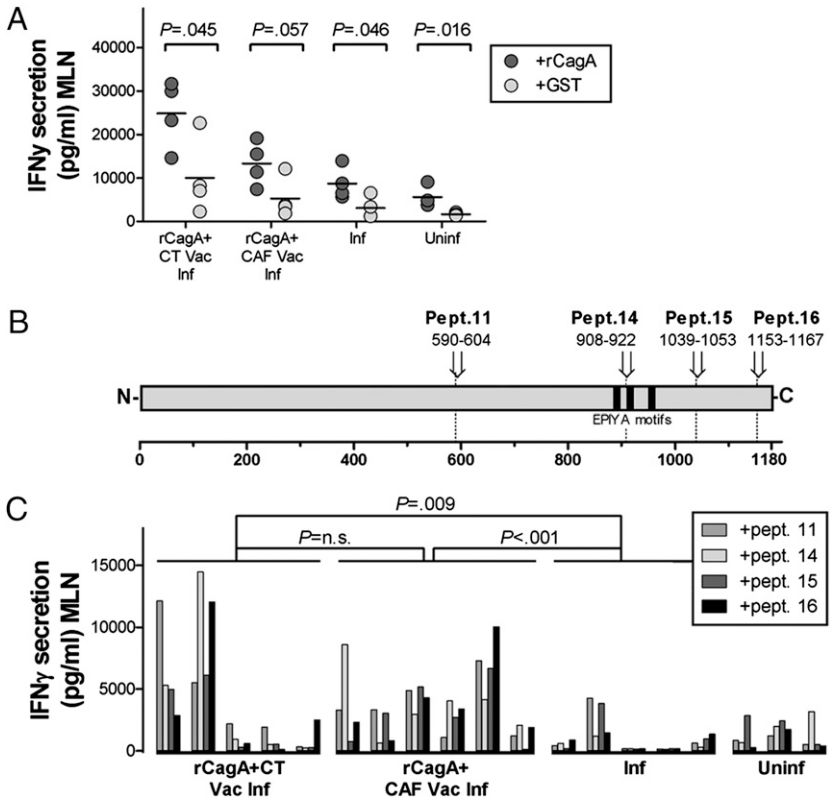


FIGURE 3. The central and C-terminal domains of CagA encode MHC class II-restricted T cell epitopes. *A–C,* MLN cultures from mice immunized and challenged as described in Fig. 2 were restimulated ex vivo for 4 d with 50 μg/ml full-length rCagA (*A*) or 10 μg/ml peptides (*C*) and assessed for IFN-γ secretion by ELISA. *B*, Schematic representation of the position of the peptides used in *C*. Data are representative of two independent experiments. n.s., not significant.

15 predicted peptides stimulated IFN-γ production in the MLN culture of at least one mouse (Fig. 3*B*, 3*C*). Most mice responded to more than one peptide; no single peptide elicited recall responses in all mice (Fig. 3*C*). Overall, the vaccinated mice of both treatment arms responded more strongly to the CagA peptides than infected-only or control mice; this reflects their overall stronger reactivity to rCagA (Fig. 3*A*). In conclusion, we show in this article that at least four distinct, nonoverlapping sequences in the C terminus of the CagA protein represent MHC class II-restricted T cell epitopes and trigger ex vivo recall responses in the draining lymph nodes.

CagA-specific T cells are sufficient to trigger gastric preneoplastic immunopathology in an adoptive transfer model

Based on our results showing that CagA-vaccinated mice respond more strongly to challenge infection than infected-only mice, and exhibit more severe gastric immunopathology, we postulated that CagA-specific T cells should be sufficient to induce gastric pathology in a host that is otherwise devoid of T cells. We used TCRβ$^{-/-}$ recipients, which cannot generate α/β^{+} T cells and are, on the one hand, incapable of controlling a *Helicobacter* infection, and on the other hand, completely protected from the T cell-driven *Helicobacter*-associated gastric immunopathology typical of immunocompetent mice (24, 27). We adoptively transferred immunomagnetically isolated, >85% pure, splenic CD4^{+}CD25^{-} T cells from CagA/CT-immunized, unchallenged mice to TCRβ$^{-/-}$ recipients, which were either experimentally infected with *H. pylori* PMSS1 on the same day or remained uninfected (Fig. 4). Additional groups of TCRβ$^{-/-}$ recipients received T cells from naive donors or from CagA/CT-immunized, challenged donors. In line with the presumed "pathogenicity" of CagA-specific T cells, the recipients of T cells isolated from CagA/CT-immunized, unchallenged mice developed extremely severe gastric pathology. This response was seen only in *H. pylori*-infected recipients; the same cells were harmless in uninfected recipients (Fig. 4*A*, 4*B*). T cells from immunized donors produced more severe pathology in infected recipients than T cells from naive donors, indicating that CagA-specific T cells must account for the infection-dependent immunopathology in the new hosts. Quite unexpectedly, the recipients of T cells from CagA-immunized mice that had been challenged with live bacteria were less pathogenic in their new hosts than cells from immunized-only mice (Fig. 4*A*, 4*B*), suggesting that an active infection in the donor suppresses T cell activity in a robust and very sustained manner. The strong pathology observed in the recipients of T cells from CagA/CT-immunized, unchallenged mice correlated with low colonization levels (Fig. 4*C*), high levels of infiltration of CD4^{+} T cells into the gastric mucosa (Fig. 4*D*), and high levels of gastric IFN-γ production (Fig. 4*E*). In contrast, the recipients of naive T cells, immunized/challenged T cells, and the uninfected recipients of immunized T cells all had relatively lower levels of gastric CD4^{+} T cell infiltration and IFN-γ production (Fig. 4*D*, 4*E*), and were colonized more heavily (Fig. 4*C*). In conclusion, the adoptive T cell transfer model revealed that CagA-specific T cells targeting the infection site in the otherwise T cell-deficient host are by themselves sufficient to produce the gastric pathology typically associated with *H. pylori* infection in immunocompetent animals, lending further support to our model that a CagA^{+} infection triggers gastric pathology through CagA's T cell antigenic properties.

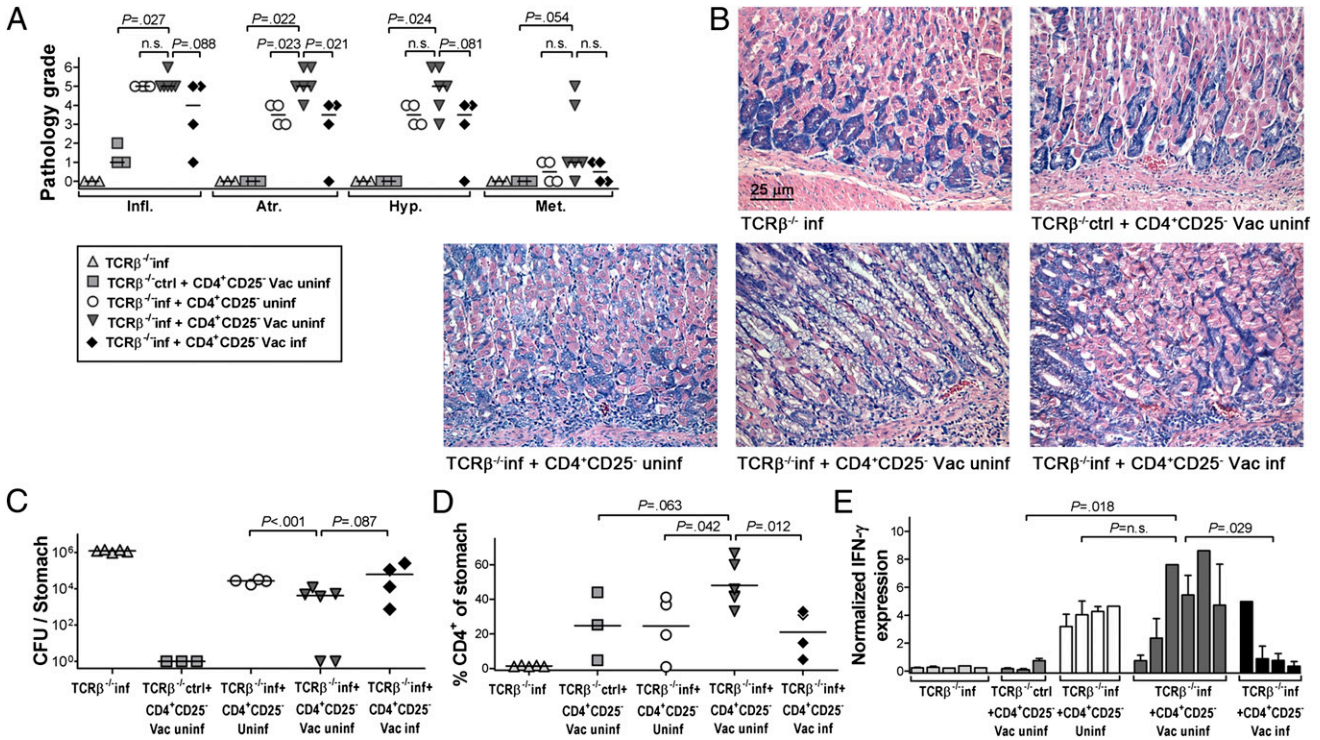


FIGURE 4. CagA-specific T cells trigger gastric preneoplastic immunopathology. Pooled splenic CD4^{+}CD25^{-} T cells were isolated from groups of mice that had been vaccinated with CagA/CT and challenged (Vac inf) or not (Vac uninf) or had remained untreated (Uninf); cells were adoptively transferred to TCR-β$^{-/-}$ recipients, which were either infected with *H. pylori* (TCR-β$^{-/-}$ inf) or remained uninfected (TCR-β$^{-/-}$ ctrl). *A–C*, Histopathology scores (*A*), representative micrographs of Giemsa-stained gastric sections (original magnification ×200) (*B*), and bacterial colonization densities (*C*) are shown for all mice 4 wk post cell transfer. *D*, Stomach-infiltrating CD4^{+} T cells as determined by FACS. *E,* Gastric IFN-γ expression as assessed by real time RT-PCR and normalized to GAPDH. *A–E*, Data are representative of two independent adoptive transfer experiments. n.s., not significant.

H. pylori-*specific vaccination promotes preneoplasia even if it confers "protective" immunity*

Vaccination with adjuvanted CagA induces local and systemic T cell responses to challenge infection that are not observed in naive mice infected for the same length of time, but nevertheless fails to confer protective immunity (Fig. 2). To assess whether a vaccination strategy conferring protective immunity sensitizes mice to enhanced gastric immunopathology, we immunized mice orally with an *H. pylori* PMSS1 whole-cell sonicate vaccine adjuvanted with CT, and challenged them with the autologous strain 2 wk after the last dose. The vaccinated mice reduced their bacterial burdens by almost two orders of magnitude compared with infected-only controls (Fig. 5*A*). However, the vaccinated mice further also developed more severe immunopathology than infected-only mice with respect to all parameters scored at 2 mo p.i. (Fig. 5*B*, 5*C*), implying that vaccination is not a suitable strategy for the prevention of gastric preneoplasia induced by $CagA^+$ strains.

Active tolerization of neonatal mice to H. pylori *prevents gastric preneoplasia*

We have shown previously that the development of immunological tolerance to *H. pylori* during the neonatal period prevents local and systemic immune responses to the pathogen and confers long-lasting protection of the host from gastritis and premalignant changes of the gastric mucosa (24). To test whether tolerization to *H. pylori* can also be achieved in the absence of active infection as an alternative strategy of gastric cancer prevention, we treated neonatal mice repeatedly with a PMSS1 whole-cell sonicate combined with anti-CD40L–neutralizing Ab during the second week of life, that is, during a time when a newborn's immune system is inherently prone to develop tolerance to self-Ags and foreign Ags (28). CD40L antagonization is used for allograft tolerization and functions by preventing costimulatory signaling during alloreactive T cell priming (29). The tolerized mice were subsequently infected as adults with live bacteria and compared with untreated mice with respect to *H. pylori* colonization, gastric T cell infiltration, and gastric histopathology (Fig. 6*A–C*). Neonatal treatment with *H. pylori* sonicate and anti-CD40L mAb was indeed sufficient to tolerize neonatal mice efficiently to the bacterium; anti-CD40L mAb-treated mice were colonized at significantly higher levels (Fig. 6*A*), exhibited lower levels of gastric T cell infiltration (Fig. 6*B*), and were protected from the gastric inflammation and associated immunopathology that are a hallmark of $CagA^+$ infection after 2 mo (Fig. 6*C*). Similar, but somewhat weaker results were obtained when rCagA was used for neonatal tolerization in conjunction with anti-CD40L mAb (Supplemental Fig. 3). Conversely, the infection-induced development of *H. pylori*-specific tolerance in neonates could be prevented by four doses of an agonistic anti-CD40 Ab administered during the first week of neonatal *H. pylori* infection; neonatally infected mice treated in this manner had significantly reduced their bacterial burdens compared with untreated, infected mice at 2 mo p.i. (Fig. 6*D*), exhibited higher levels of gastric T cell infiltration (Fig. 6*E*), and had developed significant gastritis, atrophy, and hyperplasia at this time point (Fig. 6*F*). In summary, our data suggest that the modulation of the CD40–CD40L interaction by neutralizing and/or activating Abs is a suitable method to shift the balance to either immunological tolerance or immunity, and to thereby influence disease outcome.

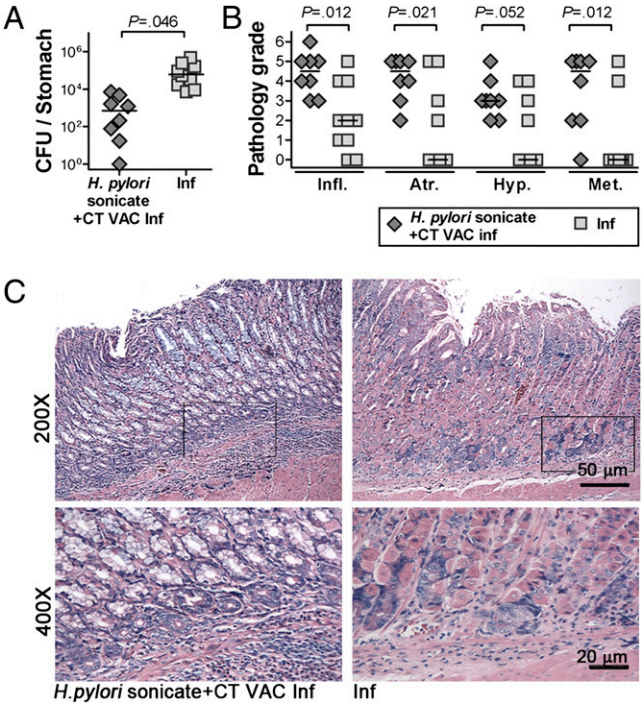


FIGURE 5. *H. pylori*-specific vaccination aggravates gastric preneoplasia. *A–C,* Mice immunized with an *H. pylori* whole-cell sonicate/CT vaccine were compared with nonimmunized counterparts 2 mo post challenge infection. *H. pylori* colonization (*A*), gastric histopathology scores (*B*), and representative low- and high-magnification micrographs of Giemsa-stained paraffin sections (*C*) are shown. Original magnification ×200 (*upper panels*) and ×400 (*lower panels*). Data are representative of three independent vaccination experiments.

Discussion

Substantial epidemiological and experimental evidence is now available to support the notion that CagA functions as a bacterial oncoprotein. A large meta-analysis of all epidemiological data available at the time showed that infection with $CagA^+$ *H. pylori* strains increases the gastric cancer risk above and beyond the risk conferred by infection alone (17). Experimental infection of Mongolian gerbils (23) and C57BL/6 mice (24) with wild type, but not CagA translocation-deficient *H. pylori* results in the rapid development of gastric cancer precursor lesions. Transgenic expression of CagA in the gastric mucosa further revealed that CagA is in itself sufficient to cause gastric hyperplasia, gastric polyps, and adenocarcinomas (22). In addition to the direct effects that the ectopic expression or natural delivery of CagA have on the cell biology of host cells, we demonstrate in this article that CagA has strong T cell antigenic properties. The central and C-terminal parts of the protein harbor in silico predicted MHC class II-restricted T cell epitopes, some of which were confirmed experimentally by ex vivo restimulation of T cells from CagA-vaccinated mice. Vaccination with CagA through two complementary routes, parenteral or mucosal, induced CagA-specific pathogenic T cells, which cause excessive gastric immunopathology in T cell-deficient recipients but fail to confer protective immunity in immunocompetent mice. Several previous findings make it seem likely that the T cell immunogenicity of CagA is at least partially responsible for CagA's oncogenic properties. First, we have shown earlier that T cells are indispensable for the induction of gastric pathology, not just in *H. pylori*, but also in *Helicobacter felis* infection models (27, 30). TCR-$\beta^{-/-}$ mice that lack functional α/β^+ T cells are protected from gastric cancer precursor lesions, and the adoptive transfer of $CD4^+CD25^-$ T cells is sufficient to trigger these lesions in resistant mice (24,

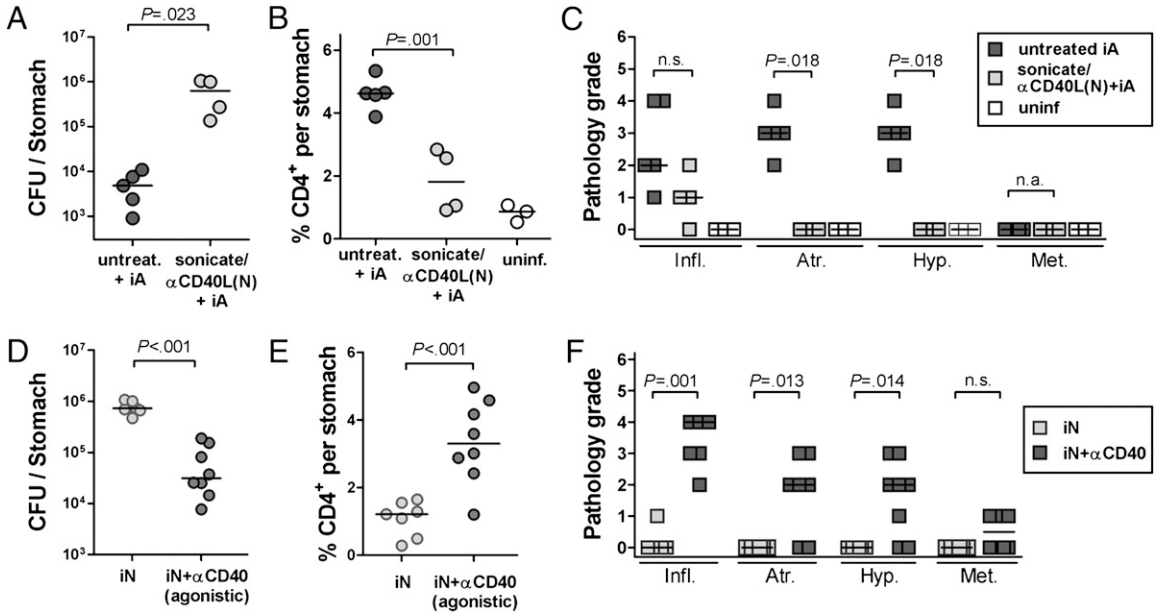


FIGURE 6. Modulation of CD40/CD40L signaling affects disease outcome. *A–C*, For the purpose of *H. pylori*-specific tolerization, neonatal mice received 4 oral doses of 50 μg *H. pylori* PMSS1 sonicate together with 50, 50, 75, and 75 μg antagonistic anti-CD40L mAb (i.p.) on days 7, 10, 12, and 14 after birth. Sonicate-treated mice and untreated control mice were infected with the autologous strain at 6 wk of age (i.e., 4 wk after the last dose) and sacrificed 8 wk later. *D–F*, Seven-day-old mice were infected with *H. pylori* PMSS1; one group received 4 i.p. doses of agonistic anti-CD40 mAb on days 0, 3, 5, and 7 p.i. (i.e., on days 7, 10, 12, and 14 after birth), whereas their littermates remained untreated. All mice were sacrificed at 8 wk p.i. *A* and *D*, *H. pylori* colonization. *B* and *E*, $CD4^+$ T cell infiltration into the gastric mucosa as determined by FACS. *C* and *F*, Histopathology scores. Uninfected, untreated control mice are shown in *B* and *C* for comparison. n.s., not significant.

27, 30). The pharmacological inhibition of T cell activation prevents and even reverses pre-existing lesions (31, 32), and neonatally infected (24) and actively tolerized mice are protected from gastric preneoplasia. Our finding that tolerant mice, despite being colonized very densely by CagA translocation-proficient bacteria, do not develop preneoplasia is perhaps the most important piece of evidence for an indirect pathogenic role of CagA (24). Finally, the results of this study provide a mechanistic explanation for our previous finding that wild type *H. pylori* and an isogenic CagE-deficient mutant differ significantly with respect to the gastric mucosal Th1 infiltration and the gastric production of IFN-γ they induce (24).

Significant progress has been made in recent years with respect to the identification of new protective *Helicobacter* Ags, understanding the mechanisms of protective immunity and the development of immunization/challenge protocols in human volunteers (33). We and others have reported previously that the challenge of whole-cell–immunized mice with avirulent mouse-adapted *H. pylori* strains such as SS1 induces more gastritis and preneoplastic lesions than infection of nonimmunized animals (27, 34, 35). In this study, we expand these findings by challenging immunized mice with a patient isolate that harbors a functional Cag pathogenicity island and is fully virulent in the murine host (24). This challenge model, which presumably mimics the human natural infection better than challenge with mouse-adapted strains, confirmed our previous results (27, 34) and demonstrated an increased susceptibility of immunized mice to gastric preneoplasia. This increased susceptibility was independent of the route of vaccine administration and of the adjuvant used, and did not correlate with a vaccination-induced reduction of the bacterial burden. Overall, the results raise the concern that any *Helicobacter* vaccine failing to achieve sterilizing immunity after challenge infection will increase rather than alleviate the vaccinee's gastric cancer risk, and will thus defy the main objective of *Helicobacter*-specific immunization.

We propose in this article that tolerization of the host to *H. pylori* may hold more promise than vaccination strategies in gastric cancer prevention. We base this proposition on our observation that neonatal mice develop natural tolerance to the bacteria if they are infected during the first 2 wk of life, at a time when the immune system is immature and inherently biased toward tolerogenic over immunogenic responses (28). Neonatally acquired, *Helicobacter*-specific tolerance is mediated and maintained by long-lived, inducible regulatory T cells, and protects the host from gastric preneoplasia not only during the neonatal period, but long into adulthood. We have expanded these findings in this study by showing that newborn mice can be tolerized actively by administration of *H. pylori* whole-cell sonicate in conjunction with anti-CD40L blocking Ab, which has been used extensively to prevent allograft rejection in preclinical models (29), and effectively prevented the gastric T cell responses and immunopathology typically associated with $CagA^+$ infection in adult-infected mice. Similar results were obtained by tolerization with CagA in conjunction with anti-CD40L Ab. The antigraft tolerization by CD40L inhibition was shown to be due to the induction of anergy in the alloreactive effector T cell pool as measured by their failure to expand and produce cytokines, but also required regulatory T cells (29). Antigraft tolerance can be broken by administration of an agonistic anti-CD40 Ab (29). Similarly, the anti-*Helicobacter* tolerance of neonatally infected mice could be broken in our hands by systemic CD40 activation.

In conclusion, we propose in this article that the increased gastric cancer risk associated with $CagA^+$ strains is due to the protein's central and C-terminally encoded T cell antigenicity. Not only do wild type *H. pylori* induce more gastric pathology than a CagA translocation-deficient isogenic mutant, but $CagA^+$ infection-associated pathology can be further exacerbated by prior immunization with rCagA. Overall, our data imply that gastric cancer management and prevention strategies will only be successful if they take into account that gastric cancer precursor lesions may be

inflammation- and/or immunity-driven rather than the direct result of bacterially induced tissue damage.

Acknowledgments

We thank Wolf-Dietrich Hardt, Jörg Hülsken, and Josef Jiricny for helpful discussions. We are grateful to Esther Kohler for expert technical assistance and to Isabella Toller, Ayca Sayi, and Vanessa Craig for assistance and discussions. We thank Wolfgang Fischer for the pGEX 4T3 expression vector and advice on CagA purification.

Disclosures

The authors have no financial conflicts of interest.

References

1. Marshall, B. J., and J. R. Warren. 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 323: 1311–1315.
2. Parsonnet, J., G. D. Friedman, D. P. Vandersteen, Y. Chang, J. H. Vogelman, N. Orentreich, and R. K. Sibley. 1991. *Helicobacter pylori* infection and the risk of gastric carcinoma. *N. Engl. J. Med.* 325: 1127–1131.
3. Parsonnet, J., S. Hansen, L. Rodriguez, A. B. Gelb, R. A. Warnke, E. Jellum, N. Orentreich, J. H. Vogelman, and G. D. Friedman. 1994. *Helicobacter pylori* infection and gastric lymphoma. *N. Engl. J. Med.* 330: 1267–1271.
4. Pritchard, D. M., and J. E. Crabtree. 2006. *Helicobacter pylori* and gastric cancer. *Curr. Opin. Gastroenterol.* 22: 620–625.
5. Frenck, R. W., Jr., and J. Clemens. 2003. *Helicobacter* in the developing world. *Microbes Infect.* 5: 705–713.
6. Gerrits, M. M., A. H. van Vliet, E. J. Kuipers, and J. G. Kusters. 2006. *Helicobacter pylori* and antimicrobial resistance: molecular mechanisms and clinical implications. *Lancet Infect. Dis.* 6: 699–709.
7. Rupnow, M. F., R. D. Shachter, D. K. Owens, and J. Parsonnet. 2001. Quantifying the population impact of a prophylactic *Helicobacter pylori* vaccine. *Vaccine* 20: 879–885.
8. Ermak, T. H., P. J. Giannasca, R. Nichols, G. A. Myers, J. Nedrud, R. Weltzin, C. K. Lee, H. Kleanthous, and T. P. Monath. 1998. Immunization of mice with urease vaccine affords protection against *Helicobacter pylori* infection in the absence of antibodies and is mediated by MHC class II-restricted responses. *J. Exp. Med.* 188: 2277–2288.
9. Satin, B., G. Del Giudice, V. Della Bianca, S. Dusi, C. Laudanna, F. Tonello, D. Kelleher, R. Rappuoli, C. Montecucco, and F. Rossi. 2000. The neutrophil-activating protein (HP-NAP) of *Helicobacter pylori* is a protective antigen and a major virulence factor. *J. Exp. Med.* 191: 1467–1476.
10. Nyström, J., and A. M. Svennerholm. 2007. Oral immunization with HpaA affords therapeutic protective immunity against *H. pylori* that is reflected by specific mucosal immune responses. *Vaccine* 25: 2591–2598.
11. Gómez-Duarte, O. G., B. Lucas, Z. X. Yan, K. Panthel, R. Haas, and T. F. Meyer. 1998. Protection of mice against gastric colonization by *Helicobacter pylori* by single oral dose immunization with attenuated *Salmonella typhimurium* producing urease subunits A and B. *Vaccine* 16: 460–471.
12. Aebischer, T., D. Bumann, H. J. Epple, W. Metzger, T. Schneider, G. Cherepnev, A. K. Walduck, D. Kunkel, V. Moos, C. Loddenkemper, et al. 2008. Correlation of T cell response and bacterial clearance in human volunteers challenged with *Helicobacter pylori* revealed by randomised controlled vaccination with Ty21a-based *Salmonella* vaccines. *Gut* 57: 1065–1072.
13. Bumann, D., W. G. Metzger, E. Mansouri, O. Palme, M. Wendland, R. Hurwitz, G. Haas, T. Aebischer, B. U. von Specht, and T. F. Meyer. 2001. Safety and immunogenicity of live recombinant *Salmonella enterica* serovar Typhi Ty21a expressing urease A and B from *Helicobacter pylori* in human volunteers. *Vaccine* 20: 845–852.
14. Malfertheiner, P., V. Schultze, B. Rosenkranz, S. H. Kaufmann, T. Ulrichs, D. Novicki, F. Norelli, M. Contorni, S. Peppoloni, D. Berti, et al. 2008. Safety and immunogenicity of an intramuscular *Helicobacter pylori* vaccine in non-infected volunteers: a phase I study. *Gastroenterology* 135: 787–795.
15. Peek, R. M., Jr., G. G. Miller, K. T. Tham, G. I. Perez-Perez, X. Zhao, J. C. Atherton, and M. J. Blaser. 1995. Heightened inflammatory response and cytokine expression in vivo to cagA+ *Helicobacter pylori* strains. *Lab. Invest.* 73: 760–770.
16. Blaser, M. J., G. I. Perez-Perez, H. Kleanthous, T. L. Cover, R. M. Peek, P. H. Chyou, G. N. Stemmermann, and A. Nomura. 1995. Infection with *Helicobacter pylori* strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. *Cancer Res.* 55: 2111–2115.
17. Huang, J. Q., G. F. Zheng, K. Sumanac, E. J. Irvine, and R. H. Hunt. 2003. Meta-analysis of the relationship between cagA seropositivity and gastric cancer. *Gastroenterology* 125: 1636–1644.
18. Odenbreit, S., J. Püls, B. Sedlmaier, E. Gerland, W. Fischer, and R. Haas. 2000. Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. *Science* 287: 1497–1500.
19. Segal, E. D., J. Cha, J. Lo, S. Falkow, and L. S. Tompkins. 1999. Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by *Helicobacter pylori*. *Proc. Natl. Acad. Sci. USA* 96: 14559–14564.
20. Amieva, M. R., R. Vogelmann, A. Covacci, L. S. Tompkins, W. J. Nelson, and S. Falkow. 2003. Disruption of the epithelial apical-junctional complex by *Helicobacter pylori* CagA. *Science* 300: 1430–1434.
21. Tan, S., L. S. Tompkins, and M. R. Amieva. 2009. *Helicobacter pylori* usurps cell polarity to turn the cell surface into a replicative niche. *PLoS Pathog.* 5: e1000407.
22. Ohnishi, N., H. Yuasa, S. Tanaka, H. Sawa, M. Miura, A. Matsui, H. Higashi, M. Musashi, K. Iwabuchi, M. Suzuki, et al. 2008. Transgenic expression of *Helicobacter pylori* CagA induces gastrointestinal and hematopoietic neoplasms in mouse. *Proc. Natl. Acad. Sci. USA* 105: 1003–1008.
23. Rieder, G., J. L. Merchant, and R. Haas. 2005. *Helicobacter pylori* cag-type IV secretion system facilitates corpus colonization to induce precancerous conditions in Mongolian gerbils. *Gastroenterology* 128: 1229–1242.
24. Arnold, I. C., J. Y. Lee, M. R. Amieva, A. Roers, R. A. Flavell, T. Sparwasser, and A. Müller. 2011. Tolerance rather than immunity protects from *Helicobacter pylori*-induced gastric preneoplasia. *Gastroenterology* 140: 199–209.
25. Davidsen, J., I. Rosenkrands, D. Christensen, A. Vangala, D. Kirby, Y. Perrie, E. M. Agger, and P. Andersen. 2005. Characterization of cationic liposomes based on dimethyldioctadecylammonium and synthetic cord factor from *M. tuberculosis* (trehalose 6,6′-dibehenate)—A novel adjuvant inducing both strong CMI and antibody responses. *Biochim. Biophys. Acta.* 1718: 22–31.
26. Agger, E. M., J. P. Cassidy, J. Brady, K. S. Korsholm, C. Vingsbo-Lundberg, and P. Andersen. 2008. Adjuvant modulation of the cytokine balance in *Mycobacterium tuberculosis* subunit vaccines; immunity, pathology and protection. *Immunology* 124: 175–185.
27. Sayi, A., E. Kohler, I. Hitzler, I. Arnold, R. Schwendener, H. Rehrauer, and A. Müller. 2009. The CD4+ T cell-mediated IFN-gamma response to *Helicobacter* infection is essential for clearance and determines gastric cancer risk. *J. Immunol.* 182: 7085–7101.
28. Arnold, B., T. Schüler, and G. J. Hämmerling. 2005. Control of peripheral T-lymphocyte tolerance in neonates and adults. *Trends Immunol.* 26: 406–411.
29. Quezada, S. A., K. Bennett, B. R. Blazar, A. Y. Rudensky, S. Sakaguchi, and R. J. Noelle. 2005. Analysis of the underlying cellular mechanisms of anti-CD154-induced graft tolerance: the interplay of clonal anergy and immune regulation. *J. Immunol.* 175: 771–779.
30. Sayi, A., E. Kohler, I. M. Toller, R. A. Flavell, W. Müller, A. Roers, and A. Müller. 2011. TLR-2-activated B cells suppress *Helicobacter*-induced preneoplastic gastric immunopathology by inducing T regulatory-1 cells. *J. Immunol.* 186: 878–890.
31. Toller, I. M., M. Altmeyer, E. Kohler, M. O. Hottiger, and A. Müller. 2010. Inhibition of ADP ribosylation prevents and cures helicobacter-induced gastric preneoplasia. *Cancer Res.* 70: 5912–5922.
32. Toller, I. M., I. Hitzler, A. Sayi, and A. Mueller. 2010. Prostaglandin E2 prevents *Helicobacter*-induced gastric preneoplasia and facilitates persistent infection in a mouse model. *Gastroenterology* 138: 1455–1467, 1467.e1–e4.
33. Velin, D., and P. Michetti. 2010. Advances in vaccination against *Helicobacter pylori*. *Expert Rev. Gastroenterol. Hepatol.* 4: 157–166.
34. Mueller, A., A. Sayi, and I. Hitzler. 2009. Protective and pathogenic functions of T-cells are inseparable during the *Helicobacter*-host interaction. *Discov. Med.* 8: 68–73.
35. Sutton, P., S. J. Danon, M. Walker, L. J. Thompson, J. Wilson, T. Kosaka, and A. Lee. 2001. Post-immunisation gastritis and *Helicobacter* infection in the mouse: a long term study. *Gut* 49: 467–473.

3.5 Caspase-1 has pro-inflammatory and regulatory properties that are differentially mediated by processing of its substrates IL-1β and IL-18

manuscript in preparation

authors: Iris Hitzler, Ayca Sayi, Esther Kohler, Wolf-Dietrich Hardt and Anne Müller

contributions: I conducted the *in vitro* experiments, the vaccination and *H. pylori* PMSS1 studies, analyzed the data and wrote the manuscript together with AM. AS and EK contributed all *H. felis* results. WDH provided vital tools.

Caspase-1 has pro-inflammatory and regulatory properties that are differentially mediated by processing of its substrates IL-1β and IL-18

Iris Hitzler[1], Ayca Sayi[1], Esther Kohler[1], Wolf-Dietrich Hardt[2], Anne Müller[1]

[1]Institute of Molecular Cancer Research, University of Zürich, Winterthurerstr. 190, Zürich, Switzerland

[2]Institute of Microbiology, Federal Institute of Technology, Zürich, Switzerland

For correspondence: Anne Müller, Institute of Molecular Cancer Research, University of Zürich, Winterthurerstr. 190, 8057 Zürich, Switzerland; email: mueller@imcr.unizh.ch; phone: ++41 44 635 3474; fax: ++41 44 635 3484

Abstract

The pro-inflammatory cysteine protease caspase-1 is autocatalytically activated upon cytosolic sensing of a variety of pathogen-associated molecular patterns by Nod-like receptors. Active caspase-1 processes pro-IL-1β and pro-IL-18 to generate the mature, bioactive cytokines and to initiate pathogen-specific immune responses. Here, we examine the role of caspase-1 and its cytokine substrates in the control and pathogenesis of chronic infection with the gram-negative gastric pathogen *Helicobacter pylori* using a variety of experimental infection, vaccine-induced protection, and gastric disease models. We show that caspase-1 activation and IL-1 signaling are absolutely required for the efficient control of *Helicobacter* infection in vaccinated mice and for the induction of *Helicobacter*-associated gastric immunopathology. IL-1R$^{-/-}$ mice fail to develop protective immunity, but are protected against *Helicobacter*-associated gastritis and gastric preneoplasia due to their inability to generate *Helicobacter*-specific Th1- and Th17 responses. In contrast, IL-18 is dispensible for vaccine-induced protective immunity, but essential for preventing excessive T-cell driven immunopathology. IL-18$^{-/-}$ animals develop strongly accelerated pathology that is accompanied by unrestricted Th17 responses. In conclusion, we show here for the first time that the processing and release of a "regulatory" caspase-1 substrate, IL-18, counteracts the pro-inflammatory activities of another caspase-1 substrate, IL-1β, thereby balancing control of the infection with the prevention of excessive gastric immunopathology.

Introduction

Cells of the innate immune system recognize microbial pathogens through their conserved molecular structures, termed 'pathogen-associated molecular patterns' (PAMPs). Examples of PAMPs include lipopolysaccharide (LPS), lipoproteins, flagellins, peptidoglycan, and microbial nucleic acids (Ishii et al. 2008). PAMPs are recognized by extracellular or endosomal, membrane-bound Toll-like receptors (TLRs) or by cytosolic Nod-like receptors (NLRs) (Brodsky et al. 2009; Franchi et al. 2009). Among the various NLRs, Nod1 and Nod2 recognize peptidoglycan metabolites and induce the transcription factor NF-κB to activate innate and adaptive immune response genes (Kim et al. 2008); in contrast, most other NLRs are involved in the assembly of a multi-protein complex called the 'inflammasome', which activates the cysteine protease caspase-1. The interaction between the NLRs and caspase-1 in the inflammasome is mediated by the bipartite adaptor protein ASC (Martinon et al., 2002). Autocatalytically activated caspase-1 processes the cytoplasmic precursors of interleukin-1β (IL-1β) and IL-18 to generate the mature, biologically active cytokines, which are subsequently released to initiate inflammation and defense mechanisms (Franchi et al. 2009; Mariathasan et al. 2007). Activation of the inflammasome is critical for the recognition of numerous gram-negative and gram-positive bacteria including *Legionella pneumophilia* (Amer et al. 2006), *Shigella flexneri* (Suzuki et al. 2007), *Pseudomonas aeruginosa* (Sutterwala et al. 2007), *Bordetella pertussis* (Dunne et al. 2010) *Salmonella enterica* (Müller et al. 2009), *Staphylococcus aureus* and *Listeria monocytogenes* (Mariathasan et al. 2006), but also for the detection and response to influenza viruses (Ichinohe et al. 2009) and alum adjuvants (Kool et al. 2008).

Several distinct inflammasomes can be distinguished based on the NLR involved. Activation of the NLRC4 inflammasome is triggered by cytosolic delivery of flagellin via type III or type IV secretion systems of gram-negative bacteria such as *S. typhimurium*, *P. aeruginosa* or *L. pneumophila* (Brodsky et al. 2009; Mariathasan et al. 2007). The NLRP3 inflammasome is activated by both foreign and endogenous molecules that include urate crystals, ATP, bacterial pore-forming toxins and particulate matter such as asbestos and silica (Brodsky et al. 2009; Franchi et al. 2009). The signals recognized by the NLRP1 inflammasome are less well understood, but seem to include anthrax lethal toxin (Boyden et al. 2006).

Persistent gastric infection with the gram-negative bacterial pathogen *Helicobacter pylori* results in chronic gastritis (Marshall et al. 1984) and predisposes carriers to a high risk of developing

gastric and duodenal ulcers, gastric cancer and gastric mucosa-associated lymphoid tissue lymphoma (Parsonnet et al. 1991; Parsonnet et al. 1994; Pritchard et al. 2006). We have shown recently that the $CD4^+$ T-cell-mediated immune response to the pathogen, rather than the presence of the bacteria per se, is a driving force behind the infection-associated gastric preneoplastic immunopathology manifesting as atrophic gastritis, epithelial hyperplasia and intestinal metaplasia (Sayi et al. 2009). The caspase-1 substrate IL-1β is highly induced in the gastric mucosa of *H. pylori*-infected individuals (Yamaoka et al. 1997) and polymorphisms associated with increased steady state levels of IL-1β predispose carriers to gastric cancer (El-Omar et al. 2000). The stomach-specific expression of human IL-1β is sufficient to induce gastric inflammation and gastric cancer in transgenic mice (Tu et al. 2008).

Surprisingly little is known about the mechanisms leading to inflammasome activation by *H. pylori,* or the NLRs involved in the process. *H. pylori* peptidoglycan appears to be detected by the NLR Nod1 in a type IV secretion system-dependent manner (Viala et al. 2004); however, Nod1 does not activate caspase-1 (Watanabe et al. 2010). Here, we have determined the role of caspase-1 and its cytokine substrates in the control and pathogenesis of *Helicobacter* infection using several complementary *Helicobacter*-induced gastric disease and colonization models. Our findings reveal dual functions for caspase-1 in *H. pylori* infection that are mediated by active IL-1β and IL-18, respectively. Whereas caspase-1-mediated IL-1β processing is absolutely required for the efficient control of *H. pylori* infection in vaccinated mice and for the induction of *Helicobacter*-associated gastric immunopathology, active IL-18 balances excessive anti-*Helicobacter* T-cell responses, prevents gastric immunopathology and promotes the development of immunological tolerance to the infection. Caspase-1 thus exhibits both pro-inflammatory and regulatory properties in the context of *Helicobacter* infection with the net result of its activation depending on the relative availability of its two cytokine substrates.

Results

H. pylori* activates caspase-1 and induces mature IL-1β and IL-18 secretion *in vitro* and *in vivo

To test whether *H. pylori* activates caspase-1 *in vitro*, we generated bone-marrow-derived dendritic cells (BMDCs), infected them o/n with *H. pylori* and quantified caspase-1 activation using the carboxyfluorescein-labeled caspase-1 substrate FAM-YVAD-FMK (FLICA). A significantly higher proportion of infected compared to uninfected cells showed evidence of caspase-1 activation under these conditions; the physical separation of the bacteria and their host cells by a transwell filter abrogated caspase-1 activation (Figure 1a). Similarly, mice that had been infected experimentally with *H. pylori* for two weeks exhibited higher numbers of $FLICA^+$ cells in their mesenteric lymph nodes, the sites of *H. pylori*-specific T-cell priming (Figure 1b).

Caspase-1 cleaves the precursor molecules of IL-1β and IL-18 to generate the mature, bioactive forms of the two cytokines. To assess whether *H. pylori* infection induces pro-IL-1β and/or pro-IL-18 expression in BMDCs *in vitro*, we quantified the transcript levels of both cytokines. Co-culture of BMDCs with *H. pylori* induced the expression of pro-IL-1β, but not of pro-IL-18 transcripts (Figure 1c). However, the release of both mature cytokines into the culture supernatant was induced by *H. pylori* infection under these conditions (Figure 1d), arguing that IL-18, but not IL-1β precursor molecules are preformed and stored in BMDCs rather than synthesized *de novo* upon infection. Similarly, the differential induction of pro-IL-1β, but not of pro-IL-18 transcripts could be detected in the gastric mucosa of *H. pylori*-infected mice (Figure 1e), despite the fact that the mature forms of both cytokines are released into the tissue (Figure 1f). Prior immunization of mice with a *Helicobacter* whole cell vaccine adjuvanted with cholera toxin further exacerbates the gastric production of both mature cytokines (Figure 1f). Taken together, the results suggest that caspase-1 is activated during *H. pylori* infection *in vitro* and *in vivo*, leading to the processing of cytokine precursors and the accumulation of mature IL-1β and IL-18 at the site of infection.

Caspase-1 is required for vaccine-induced protective immunity against *H. pylori* infection and controls excessive gastric immunopathology

Having observed that *H. pylori* infection efficiently activates caspase-1 *in vitro* and *in vivo*, we sought to elucidate the effects of caspase-1 gene deletion in mouse models of vaccine-induced protective immunity and in experimental infection models. We orally immunized wild type and

caspase-1$^{-/-}$ mice with three consecutive weekly doses of the above-mentioned *H. pylori* whole cell sonicate vaccine adjuvanted by cholera toxin, and determined the outcome of autologous challenge infection in the vaccinated compared to naive mice of both strains. Immunized caspase-1$^{-/-}$ mice were significantly less able to control the challenge infection than wild type controls (Figure 2a) and also had lower levels of gastric leucocyte and CD4^{+} T-cell infiltration (supplementary Figure 1), two parameters known to represent independent correlates of vaccine-induced protection (Hitzler et al., 2011). Surprisingly, the gastric expression of IFN-γ and IL-17 was normal in the vaccinated caspase-1$^{-/-}$ animals relative to their wild type counterparts (Figure 2b,c). In contrast to their apparent defect in developing protective immunity upon vaccination, naive caspase-1$^{-/-}$ mice appeared to better control the experimental infection than their naive wild type counterparts (Figure 2a); the lower bacterial burden of naive caspase-1$^{-/-}$ mice correlated with their strongly elevated levels of gastric IL-17 (Figure 2c), which hardly differed from that of their vaccinated littermates.

To further elucidate the outcome of experimental infection in a *Helicobacter*-dependent gastric disease model, caspase-1$^{-/-}$ and wild type mice were infected for one or three months with the close *H. pylori* relative *H. felis*, a strain that is known to colonize mice persistently and to efficiently induce chronic gastritis and gastric preneoplastic pathology (Sayi et al., 2009; Toller et al., 2010a,b). In this model, caspase-1$^{-/-}$ mice were also colonized at significantly lower levels than wild type controls at the two time points investigated post infection, i.e. they were able to control the infection more efficiently (Figure 2d, supplemental Figure 2a). As a consequence, the caspase-1$^{-/-}$ strain exhibited substantially more gastric inflammation and preneoplastic immunopathology -manifesting histologically as gastric atrophy, epithelial hyperplasia and intestinal metaplasia- than wild type mice at both time points (Figure 2e,f, supplemental Figure 2b,c). The lower bacterial burden and increased immunopathology of the caspase-1$^{-/-}$ strain coincided with higher gastric expression of IL-17, but not of IFN-γ relative to wild type mice (Figure 2g,h). To determine whether the stronger immune response to *H. pylori* and the associated aggravated immunopathology of caspase-1$^{-/-}$ mice is a phenotype linked to the hematopoietic system, we irradiated wild type mice and reconstituted their hematopoietic system with bone marrow from wild type or caspase-1$^{-/-}$ mice. Only the recipients of caspase-1$^{-/-}$ bone marrow developed gastric pathology in an infection-dependent manner, indicating that the increased susceptibility of caspase-1$^{-/-}$ mice is a consequence of the caspase-1 defect in the hematopoietic system (data not shown). In summary, the results suggest that caspase-1 plays a

dual role in the context of *H. pylori* infection. On the one hand, its activity is required for vaccine-induced protective immunity against *H. pylori*; on the other hand, caspase-1 prevents excessive immune activation against *H. pylori* infection in naive mice and thereby restricts infection-associated gastric immunopathology.

The caspase-1 substrate IL-1β, but not IL-18, is required for vaccine-induced protective immunity to *H. pylori*

As mentioned before, caspase-1 cleaves the precursor molecules of IL-1β and IL-18 to generate the mature, bioactive forms of the two cytokines. Cytokine-independent effects of caspase-1 have also been described. To determine whether the postulated pro-inflammatory and regulatory activities of caspase-1 require IL-1β and/or IL-18 -or neither of the two- we first investigated the phenotypes of genetically modified strains lacking either the IL-1β receptor (IL-1R) or IL-18 in the vaccination/challenge model. Interestingly, whereas IL-1R$^{-/-}$ animals exhibited a clear defect in generating vaccine-induced protective immunity, IL-18$^{-/-}$ mice were protected as well as wild type animals (Figure 3a). Several correlates of protection, including the gastric infiltration of CD45^{+} leucocytes, CD4^{+} T-cells, c-Kit^{+} mast cells, and Ly6G^{+} neutrophils, confirmed the defect of the IL-1R$^{-/-}$, but not of the IL-18$^{-/-}$ strain, in generating protective immunity (Figure 3b-e). The complete lack of protection in IL-1R$^{-/-}$ animals was reflected in their strongly reduced gastric IFN-γ and IL-17 expression levels (Figure 3f,g); intracellular cytokine staining revealed that the infiltration of CD4^{+} IFN-γ^{+} Th1 cells and CD4^{+} IL-17^{+} Th17 cells was indeed impaired in IL-1R$^{-/-}$ animals (data not shown). In contrast, the well-protected IL-18$^{-/-}$ mice exhibited normal gastric expression of IL-17, and a relatively modest defect in IFN-γ expression (Figure 3f,g). As control of the challenge infection in naive (as opposed to vaccinated) caspase-1$^{-/-}$ mice was more efficient than in the wild type animals (Figure 2b), we also examined and compared the colonization levels in naive mice of the IL-1R$^{-/-}$ and IL-18$^{-/-}$ backgrounds to wild type controls. Interestingly, whereas the IL-1R$^{-/-}$ strain was unable to spontaneously reduce the colonization levels compared to wild type mice, the IL-18$^{-/-}$ animals phenocopied the caspase-1$^{-/-}$ strain (Figure 3A). As observed for the caspase-1$^{-/-}$ mice, the spontaneous reduction of the challenge infection in naive IL-18$^{-/-}$ mice correlated with strongly enhanced gastric expression of IL-17 (Figure 3g). Taken together, the results suggest that caspase-1 and its substrate IL-1β but not IL-18, are required for the protective, Th1/Th17-driven immunity against *H. pylori* that is conferred by a whole cell sonicate vaccine. Conversely, the spontaneous control of the challenge

infection in naive caspase-1$^{-/-}$ and IL-18$^{-/-}$ mice suggests that the regulatory (but not the pro-inflammatory) properties of caspase-1 require mature IL-18.

IL-18, but not IL-1R signaling, is required for the regulatory activities of caspase-1

Based on our observation that naive caspase-1$^{-/-}$ and IL-18$^{-/-}$ mice are better able than wild-type animals to spontaneously control *H. pylori* infection -possibly due to their dysregulated gastric IL-17 responses- we asked whether IL-18 would also be required to restrict immunopathology in the *H. felis*-induced disease model introduced above. Indeed, IL-18$^{-/-}$ animals exhibited a significantly enhanced susceptibility to *H. felis*-induced gastric inflammation, atrophy, epithelial hyperplasia and intestinal metaplasia compared to wild type animals, thus phenocopying the effects of caspase-1 gene deletion (Figure 4a,b). The enhanced and accelerated immunopathology of *H. felis*-infected IL-18$^{-/-}$ mice was associated with lower *H. felis* colonization levels (Figure 4c), and higher gastric expression of IL-17 (but not IFN-γ; Figure 4d,e), suggesting that the increased susceptibility of both the caspase-1$^{-/-}$ and IL-18$^{-/-}$ strains is a consequence of excessive Th17-cell-driven immune responses to the pathogen. In contrast, IL-1R$^{-/-}$ mice did not show enhanced susceptibility to *H. felis*-induced gastric immunopathology (Figure 4a,b), did not control *H. felis* colonization levels better than wild type animals (Figure 4c), and also did not express more gastric IL-17 and IFN-γ (Figure 4d,e). In fact, not only were IL-1R$^{-/-}$ mice not as hyper-susceptible to gastric immunopathology as the caspase-1$^{-/-}$ and IL-18$^{-/-}$ strains; they were even protected from the gastritis and gastric preneoplasia that arises in wild type mice after extended periods (3 months) of experimental infection with *H. felis* (Figure 4f,g). The resistance of IL-1R$^{-/-}$ animals to gastric pathology correlated well with their higher bacterial loads (Figure 4h) and their lower gastric expression of IL-17 and IFN-γ (Figure 4i,j). In conclusion, the results from the *H. felis*-induced disease model imply that the regulatory properties of caspase-1 (which restrict the spontaneous control of *H. pylori* and *H. felis* infection and limit gastric immunopathology) depend on the processing of IL-18, whereas the pro-inflammatory/ pathogenic properties of caspase-1 are mediated via IL-1R. Taken together, the combined results from the vaccination and disease models strongly imply that caspase-1 has both pro-inflammatory and regulatory activities, and these depend on the availability of its two alternative substrates, IL-1β and IL-18.

Discussion

Autocatalytic caspase-1 activation by the inflammasome is essential for the control of numerous gram-negative and gram-positive bacteria. To elucidate the role of caspase-1, and of its two main substrates IL-1β and IL-18, in the control and pathogenesis of *Helicobacter* infection, we comparatively analyzed the phenotypes of respective gene-targeted mice with regard to vaccine-induced protective immunity and infection-associated gastric preneoplastic pathology. Our findings confirm and extend previous results demonstrating a direct role of IL-1 signaling in the induction of gastritis and gastric carcinogenesis (Tu et al. 2008); IL-1R$^{-/-}$ mice were completely protected against infection-induced gastritis and showed no evidence of preneoplastic pathology in our *H. felis*-induced disease model. We attribute the phenotype of IL-1R$^{-/-}$ mice to their inability to generate Th1- and Th17-polarized T-cell responses to the infection, which is evident in experimental infection models and, even more strikingly, in vaccination/challenge models. Consequently, IL-1R$^{-/-}$ mice are incapable of spontaneously controlling *Helicobacter*, and also fail to generate protective immunity upon *Helicobacter*-specific immunization. Our results support a crucial role for IL-1 signaling in the differentiation of Th1 and Th17 cell subsets, both of which are essential for the development of protective immunity on the one hand, and gastric CD4^{+} T-cell-driven immunopathology on the other (Akhiani et al. 2002; Sayi et al. 2009; Velin et al. 2009).

IL-18$^{-/-}$ mice show the completely opposite phenotype: they develop normal protective immunity to *H. pylori* under conditions of vaccination followed by challenge infection, despite a somewhat reduced ability to generate Th1-polarized effector T-cell responses; our results are thus in agreement with a previously published study (Panthel et al. 2003). Furthermore, naive IL-18$^{-/-}$ mice are capable of spontaneously controlling experimental infection with *H. pylori* SS1, PMSS1 or *H. felis*, thereby phenocopying caspase-1$^{-/-}$ animals and exhibiting striking similarity to mice in which regulatory T-cells have been depleted quantitatively (Sayi et al. 2011). The spontaneous control of the infection in IL-18$^{-/-}$ and caspase-1$^{-/-}$ mice coincides with their strongly elevated gastric IL-17 levels and severely enhanced and accelerated gastric preneoplastic pathology. In summary, the lack of IL-18 (in IL-18$^{-/-}$ mice) or of its processing (in caspase-1$^{-/-}$ mice) impairs Th1 responses on the one hand, as shown previously (Fantuzzi et al. 1998; Gu et al. 1997), but also has clear implications for immunoregulatory processes, with the latter effects driving the *Helicobacter*-associated phenotypes of the respective gene-targeted strains. Our

results are somewhat in line with two recent studies demonstrating a protective role for IL-18, produced upon activation of the NLRP3 inflammasome, in DSS-induced colitis (Dupaul-Chicoine et al. 2010; Zaki et al. 2010). In this model, lack of expression of NLRP3, Caspase-1 and ASC in intestinal epithelial cells rendered mice hypersusceptible to DSS-induced colitis; this phenotype could at least partially be reversed by administration of recombinant IL-18 (Dupaul-Chicoine et al. 2010; Zaki et al. 2010). One study attributed the protective activity of IL-18 to its putative beneficial effects on enterocyte proliferation and maintenance of epithelial integrity (Zaki et al. 2010). In contrast, the enhanced gastric IL-17 expression and lower *Helicobacter* colonization that are a hallmark of infected IL-18$^{-/-}$ and caspase-1$^{-/-}$ mice suggest that IL-18 is required for the (Treg-mediated) suppression of pathogenic Th17 responses in our models.

Results from other colitis models are in conflict with the notion that inflammasome activation mediates protection from disease. For instance, gut inflammation induced by *Salmonella typhimurium* infection depends on the type III secretion system-dependent activation of the NLRC4 inflammasome in stromal cells (Müller et al. 2009). Older publications have highlighted a crucial contribution of inflammasome/caspase-1 activation and IL-18 production by hematopoietic cells to both DSS-induced and TNBS-induced colitis (Kanai et al. 2001; Siegmund et al. 2001). All studies combined suggest that gastrointestinal inflammation induced by microbial pathogens or the disruption of epithelial barrier functions is influenced greatly by at least four independent parameters: the (predominant) NLR sensing the PAMPs or danger signals, the cell type in which inflammasome activation occurs, the target cell type of the caspase-1 substrates, and, -in our model- the relative availability of pro-IL-1β and pro-IL-18.

Our preliminary bone marrow transfer experiments imply that caspase-1 expression in hematopoietic cells, rather than in stromal or epithelial cells, directs the phenotype of caspase-1$^{-/-}$ and IL-18$^{-/-}$ mice. Therefore, we suggest that caspase-1 activation and subsequent IL-18 secretion by immune cells, possibly in response to as yet uncharacterized *Helicobacter*-specific PAMPs or danger signals secreted by the infected epithelium, balance the pro-inflammatory activities of simultaneously produced mature IL-1β, and thereby prevent excessive pathogenic Th17 responses and gastric preneoplastic immunopathology.

Methods

Animal experimentation

C57BL/6 wild-type, caspase $1^{-/-}$, IL-$1R^{-/-}$ and IL-$18^{-/-}$ mice were originally purchased from Charles River Laboratories (Sulzfeld, Germany). All mice were bred at a University of Zurich specific pathogen-free facility. Mice were housed in individually ventilated cages and mixed gender groups were included in studies at 5–6 weeks of age. All animal experimentation was reviewed and approved by the Zurich cantonal veterinary office. For immunization, mice received 500µg of *H. pylori* SS1 whole cell sonicate along with 10µg of CT (List Biologicals, Campbell, CA) by oral gavage in a total volume of 200µl three times at weekly intervals. Two weeks after the last immunization, immunized and naive control mice were infected with 10^8 CFU of *H. pylori* SS1 (Lee et al. 1997) grown as described previously (Sayi et al. 2009). Mice were sacrificed 2 weeks after challenge. Bacterial colonization was assessed by colony count assay as described earlier (Sayi et al. 2009) to determine colony forming units (CFU) per stomach. For the purpose of *H. felis* infection, mice were infected with two consecutive orogastric doses of *H. felis* grown as described (Sayi et al. 2009). At study endpoints, stomachs were retrieved and dissected longitudinally into several equally sized pieces. For the quantitative PCR-based assessment of *H. felis* colonization, genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and subjected to quantitative PCR analysis of the *fla*B gene, as described (Sayi et al. 2009). For the quantitative assessment of gastric histopathology, Giemsa-stained paraffin-embedded stomach sections were scored on a scale of 0–6 for the parameters of chronic inflammation, atrophy, epithelial hyperplasia, and metaplasia, as described in detail previously (Sayi et al. 2009). All pictures were taken with a 203 magnification on a Leica Leitz DM RB microscope equipped with a DFC 420C camera. Images were acquired using the Leica Application Suite 3.3.0 software. Scale bars indicate 50 µm.

Preparation of BMDCs, MLN single cell suspensions and assessment of caspase-1 activation

For generation of BMDCs a single cell suspension was prepared from bone marrow isolated from the hind legs of a donor mouse. 200k cells/well were seeded in 24-well plates in medium containing 10ng/ml GM-CSF, after 4 days fresh medium was added and after 7 days the cells were used for experiments. Mesenteric lymph nodes of single mice were digested in 1mg/ml collagenase (Sigma-Aldrich) for 45 min at 37°C with shaking prior to filtering through a cell strainer (40µm; BD Biosciences, San Jose, CA). In order to assess caspase-1 activation, cells were stained with a carboxyfluorescein-labeled caspase-1-specific FLICA inhibitor (FAM-YVAD-FMK; ImmunoChemistry Technologies, Bloomington, MN) according to the manufacturer's instructions and analyzed on a flow cytometer. Dead cells were excluded by gating out propidium iodide (PI) positive cells. Cytokine secretion was measured using ELISA kits for IL-1β (BD

Biosciences) and IL-18 (MBL, Naka-ku Nagoya, Japan) according to the manufacturers' instructions with appropriate dilutions of cell culture supernatants or normalized amounts of gastric protein extracts.

Preparation of gastric single cell suspensions and flow cytometry

One-sixth of every stomach (antrum and corpus) and corresponding mesenteric lymph nodes were digested in 1mg/ml collagenase for 30 min at 37°C with shaking prior to mechanical disruption between glass slides and filtering through a cell strainer (40µm). Single cell suspensions were stained directly for FACS analysis. The following antibodies were used: CD4-FITC, CD4-APC (BD Biosciences), CD45-PB, Ly6G-APC, rat anti-mouse CD117 (all BioLegend, San Diego, CA) followed by goat anti-rat FITC (Sigma-Aldrich). IFN-γ-PE-Cy7 (BD), IL-17-APC and FoxP3-APC (both eBioscience, San Diego, CA) were used for intracellular staining. Prior to intracellular cytokine staining, cells were stimulated and blocked in medium containing 2.5µg/ml Brefeldin A (AppliChem, Darmstadt, Germany), 0.2µM ionomycin (Santa Cruz Biotechnology, Santa Cruz, USA) and 50ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich) for 5h, stained for extracellular markers and fixed in 4% paraformaldehyde. Flow cytometry was performed on a Cyan ADP 9 instrument (Beckman Coulter, Brea, CA) and analyzed using FlowJo software (TreeStar, Ashland, USA).

Real time RT-PCR

For real-time RT-PCR, total RNA was isolated from one-sixth of every stomach (antrum and corpus) using NucleoSpin RNA II kits (Macherey-Nagel, Düren, Germany). The corresponding cDNA served as a template for real-time PCR performed using the LightCycler 480 SYBR Green I master kit (Roche, Basel, Switzerland). Absolute values of IFN-γ, IL-17, IL-1β and IL-18 expression were normalized to GAPDH expression (conditions: Tm 55°C, 50 cycles; primers: GAPDH fw GAC ATT GTT GCC ATC AAC GAC C / GAPDH rv CCC GTT GAT GAC CAG CTT CC, IFN-γ fw CAT GGC TGT TTC TGG CTG TTA CTG / IFN-γ rv GTT GCT GAT GGC CTG ATT GTC TTT, IL-17 fw GCT CCA GAA GGC CCT CAG A / IL-17 rv AGC TTT CCC TCC GCA TTG A, IL-1β fw TTG ACG GAC CCC AAA AGA TG / IL-1β rv TGG ACA GCC CAG GTC AAA G, IL-18 fw ACT GTA CAA CCG CAG TAA TAC / IL-18 rv AGT GAA CAT TAC AGA TTT ATC CC).

Treg conversion assay

$CD4^+CD25^-$ T-cells were prepared from single-cell suspensions of uninfected C57BL/6 spleens by immunomagnetic sorting (R&D Systems). BMDCs and T-cells were co-cultured at a ratio of 1:2 ($0.5x10^5$ DC to$1x10^5$ T-cells) in RPMI containing 10% FCS, 10ng/ml rTGF-β (PeproTech, Rocky Hill, NJ), 10ng/ml rIL-2 (R&D Systems) and 1µg/ml anti-CD3ε (BD Bioscience). After 72h of co-culture, the cells were stained first for CD4 and then, after fixation, for FoxP3. The percentage of $FoxP3^+$ $CD4^+$ T-cells was assessed by FACS analysis.

Statistics

GraphPad Prism (GraphPad Software, La Jolla, USA) was used for statistical analyses. The significance of categorical differences in histopathology scores was calculated by a Mann–Whitney or unpaired Wilcox test, and the significance of numerical differences was calculated by Student's t-test. In column bar graphs, SEMs of the mean are indicated by vertical bars, n.s. stands for not significant.

References

Akhiani AA, et al. 2002. Protection Against Helicobacter pylori Infection Following Immunization Is IL-12-Dependent and Mediated by Th1 Cells. J Immunol. 169:6977-6984.

Amer A, et al. 2006. Regulation of Legionella Phagosome Maturation and Infection through Flagellin and Host Ipaf. Journal of Biological Chemistry. 281:35217-35223.

Boyden ED, Dietrich WF. 2006. Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. Nat Genet. 38:240-244.

Brodsky IE, Monack D. 2009. NLR-mediated control of inflammasome assembly in the host response against bacterial pathogens. Semin Immunol. 21:199-207.

Dunne A, et al. 2010. Inflammasome Activation by Adenylate Cyclase Toxin Directs Th17 Responses and Protection against Bordetella pertussis. The Journal of Immunology. 185:1711-1719.

Dupaul-Chicoine J, et al. 2010. Control of Intestinal Homeostasis, Colitis, and Colitis-Associated Colorectal Cancer by the Inflammatory Caspases. Immunity. 32:367-378.

El-Omar EM, et al. 2000. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature. 404:398-402.

Fantuzzi G, et al. 1998. Interleukin-18 regulation of interferon gamma production and cell proliferation as shown in interleukin-1beta-converting enzyme (caspase-1)-deficient mice. Blood. 91:2118-2125.

Franchi L, Eigenbrod T, Munoz-Planillo R, Nunez G. 2009. The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. Nat Immunol. 10:241-247.

Gu Y, et al. 1997. Activation of interferon-gamma inducing factor mediated by interleukin-1beta converting enzyme. Science. 275:206-209.

Ichinohe T, et al. 2009. Inflammasome recognition of influenza virus is essential for adaptive immune responses. The Journal of Experimental Medicine. 206:79-87.

Ishii KJ, et al. 2008. Host innate immune receptors and beyond: making sense of microbial infections. Cell Host Microbe. 3:352-363.

Kanai T, et al. 2001. Macrophage-Derived IL-18-Mediated Intestinal Inflammation in the Murine Model of Crohn's Disease. Gastroenterology. 121:875-888.

Kim YG, et al. 2008. The cytosolic sensors Nod1 and Nod2 are critical for bacterial recognition and host defense after exposure to Toll-like receptor ligands. Immunity. 28:246-257.

Kool M, et al. 2008. Cutting Edge: Alum Adjuvant Stimulates Inflammatory Dendritic Cells through Activation of the NALP3 Inflammasome. J Immunol. 181:3755-3759.

Lee A, et al. 1997. A standardized mouse model of Helicobacter pylori infection: introducing the Sydney strain. Gastroenterology. 112:1386–1397.

Mariathasan S, Monack DM. 2007. Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation. Nat Rev Immunol. 7:31-40.

Mariathasan S, et al. 2006. Cryopyrin activates the inflammasome in response to toxins and ATP. Nature. 440:228-232.

Marshall BJ, Warren JR. 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet. 1:1311-1315.

Müller AJ, et al. 2009. The S. Typhimurium Effector SopE Induces Caspase-1 Activation in Stromal Cells to Initiate Gut Inflammation. Cell Host & Microbe. 6:125-136.

Panthel K, Faller G, Haas R. 2003. Colonization of C57BL/6J and BALB/c Wild-Type and Knockout Mice with Helicobacter pylori: Effect of Vaccination and Implications for Innate and Acquired Immunity. Infect. Immun. 71:794-800.

Parsonnet J, et al. 1991. Helicobacter pylori infection and the risk of gastric carcinoma. N Engl J Med. 325:1127-1131.

Parsonnet J, et al. 1994. Helicobacter pylori infection and gastric lymphoma. N Engl J Med. 330:1267-1271.

Pritchard DM, Crabtree JE. 2006. Helicobacter pylori and gastric cancer. Curr Opin Gastroenterol. 22:620-625.

Sayi A, et al. 2009. The $CD4^+$ T Cell-Mediated IFN-gamma Response to Helicobacter Infection Is Essential for Clearance and Determines Gastric Cancer Risk. J Immunol. 182:7085-7101.

Sayi A, et al. 2011. TLR-2-activated B cells suppress Helicobacter-induced preneoplastic gastric immunopathology by inducing T regulatory-1 cells. J Immunol. 186:878-890.

Siegmund B, Lehr H-A, Fantuzzi G, Dinarello CA. 2001. IL-1beta-converting enzyme (caspase-1) in intestinal inflammation. Proceedings of the National Academy of Sciences of the United States of America. 98:13249-13254.

Sutterwala FS, et al. 2007. Immune recognition of Pseudomonas aeruginosa mediated by the IPAF/NLRC4 inflammasome. The Journal of Experimental Medicine. 204:3235-3245.

Suzuki T, et al. 2007. Differential Regulation of Caspase-1 Activation, Pyroptosis, and Autophagy via Ipaf and ASC in *Shigella*-Infected Macrophages. PLoS Pathog. 3:e111.

Tu S, et al. 2008. Overexpression of Interleukin-1 beta Induces Gastric Inflammation and Cancer and Mobilizes Myeloid-Derived Suppressor Cells in Mice. Cancer Cell. 14:408-419.

Velin D, et al. 2009. Interleukin-17 Is a Critical Mediator of Vaccine-Induced Reduction of Helicobacter Infection in the Mouse Model. Gastroenterology. 136:2237-2246.e2231.

Viala J, et al. 2004. Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. Nat Immunol. 5:1166-1174.

Watanabe T, et al. 2010. NOD1 contributes to mouse host defense against Helicobacter pylori via induction of type I IFN and activation of the ISGF3 signaling pathway. The Journal of Clinical Investigation. 120:1645-1662.

Yamaoka Y, et al. 1997. Induction of various cytokines and development of severe mucosal inflammation by cagA gene positive Helicobacter pylori strains. Gut. 41:442-451.

Zaki MH, et al. 2010. The NLRP3 Inflammasome Protects against Loss of Epithelial Integrity and Mortality during Experimental Colitis. Immunity. 32:379-391.

Figures

Figure 1

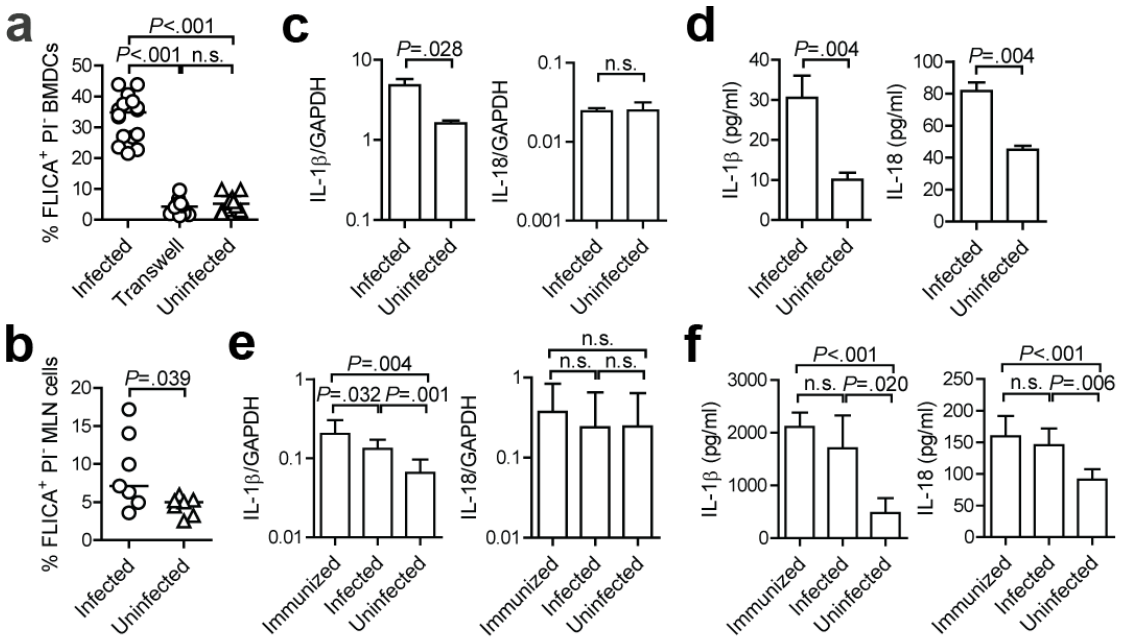


Figure 1. Caspase-1 activation and IL-1β/IL-18 secretion are induced by *H. pylori* infection *in vitro* and *in vivo.*

(**a,b**) Percentages of *H. pylori*-infected and uninfected bone marrow-derived dendritic cells (BMDCs) (**a**) and total MLN cells isolated from infected or uninfected donor mice (**b**) positive for activated caspase-1 as determined by FLICA staining. Horizontal lines represent median values. (**c,e**) Expression of pro-IL-1β and IL-18 transcript in infected and uninfected BMDCs (**c**) and the gastric mucosa (**e**) of immunized & infected (imm/inf), infected or uninfected mice, respectively, as determined by real-time RT-PCR and normalized to GAPDH. Data are shown as mean +/- SEM. (**d,f**) IL-1β and IL-18 secretion as determined by ELISA of the supernatants of infected and uninfected BMDCs (**d**) and the gastric mucosa (**f**) of immunized & infected (imm/inf), infected or uninfected mice.

Figure 2

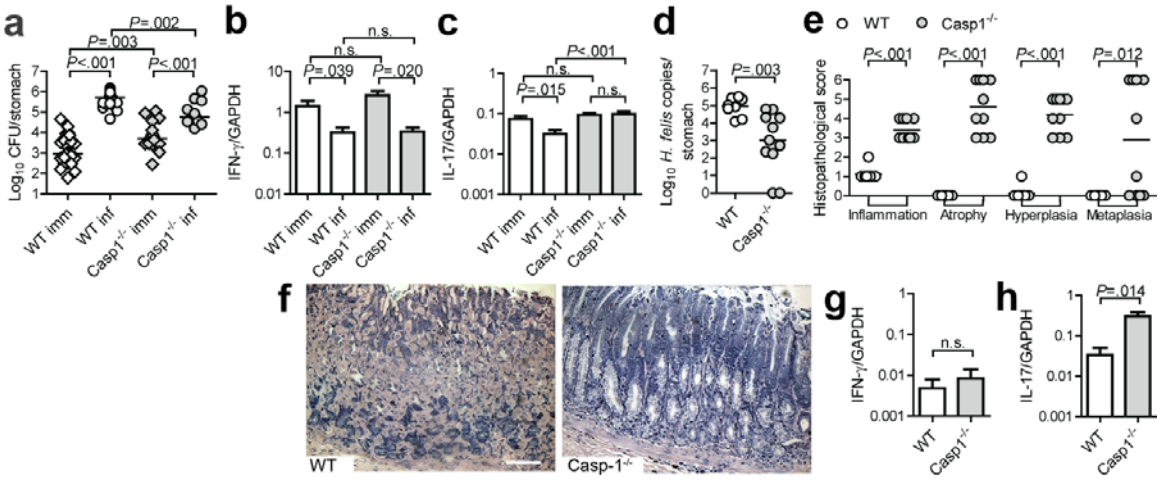


Figure 2. Absence of caspase-1 hampers vaccine-induced protective immunity against *H. pylori* infection and exacerbates gastric immunopathology

(**a**) *H. pylori* colonization levels as assessed by colony count assay (CFU = colony forming units) for immunized & challenged (imm/inf) and infected (inf) wild type and caspase-1$^{-/-}$ mice. Horizontal lines represent median values. (**b,c**) Real-time PCR results for IFN-γ (**b**) and IL-17 (**c**) expression in the gastric mucosa of the mice shown in **a**, normalized to GAPDH (mean +/- SEM). (**d**) Colonization levels as determined by quantitative PCR of the *flaB* gene of wild type and caspase-1$^{-/-}$ mice infected with *H. felis* for 1 month; medians are indicated by horizontal lines. (**e**) Histopathology scores assigned to every mouse shown in **d** for all indicated parameters on a scale of 0-6. Horizontal lines indicate the means. (**f**) Representative micrographs of Giemsa-stained sections of one *H. felis*-infected wild type and caspase-1$^{-/-}$ mouse, respectively. (**g,h**) Real-time PCR results for gastric IFN-γ (**g**) and IL-17 (**h**) expression in the same groups of mice, normalized to GAPDH.

Figure 3

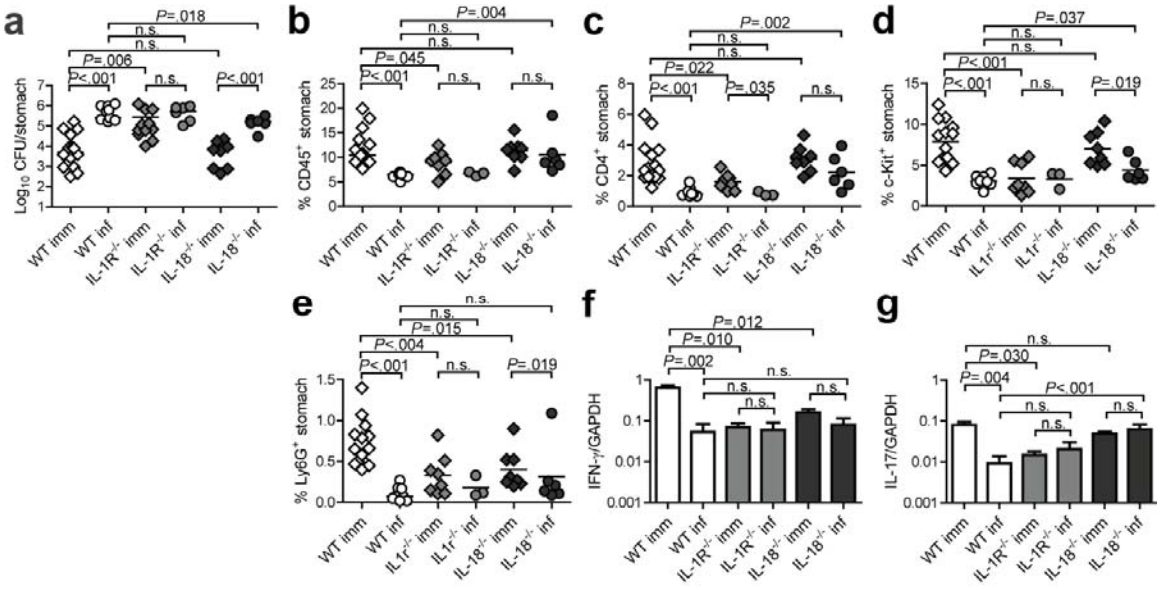


Figure 3. Vaccine-induced protective immunity to *H. pylori* relies on the caspase-1 substrate IL-1β, but not IL-18

(**a**) *H. pylori* colonization levels as assessed by colony count assay for immunized & challenged (imm/inf) and infected (inf) wild type, IL-1R$^{-/-}$ and IL-18$^{-/-}$ mice. (**b-e**) Percentages with regard to total stomach cells of leucocytes (CD45^{+}) (**b**), CD4^{+} T cells (**c**), mast cells (c-Kit^{+}) (**d**), and neutrophils (Ly6-G^{+}) (**e**) infiltrating the gastric mucosa of the mice shown in **a**. Horizontal lines represent median values. (**f,g**) Real-time PCR results for gastric IFN-γ (**f**) and IL-17 (**g**) expression in the same mice, normalized to GAPDH.

Figure 4

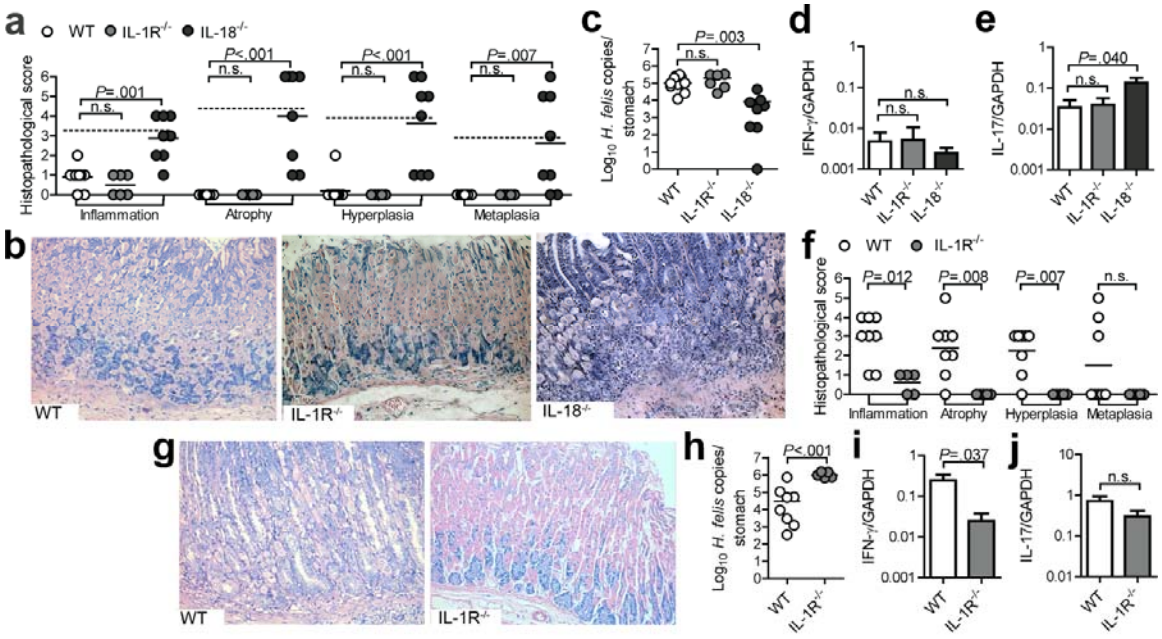
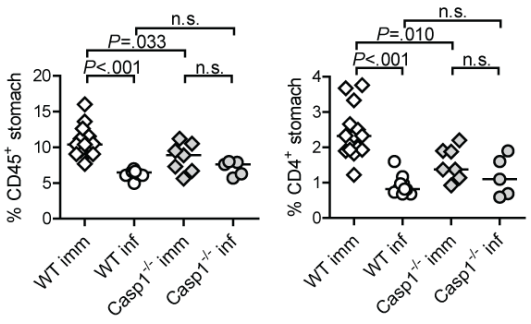


Figure 4. The regulatory activities of caspase-1 require IL-18 but not IL-1R

(**a**) Histopathology scores assigned to wild type, IL-1R$^{-/-}$ and IL-18$^{-/-}$ mice infected with *H. felis* for 1 month. Horizontal lines indicate the means, dashed lines represent the mean values for caspase-1$^{-/-}$ mice as seen in Fig. 2e. (**b**) Representative micrographs of Giemsa-stained sections of one mouse per group. (**c**) *H. felis* colonization as determined by quantitative PCR of the *flaB* gene; medians are indicated by horizontal lines. (**d,e**) Real-time PCR results for IFN-γ (**d**) and IL-17 (**e**) expression in the gastric mucosa, normalized to GAPDH. (**f,g**) Histopathology scores and representative micrographs of wild type and IL-1R$^{-/-}$ mice infected with *H. felis* for 3 months. Horizontal lines indicate the means. (**h**) *H. felis* colonization as determined by quantitative PCR of the *flaB* gene; medians are indicated by horizontal lines. (**i,j**) Real-time PCR results for gastric IFN-γ (**i**) and IL-17 (**j**) expression, normalized to GAPDH.

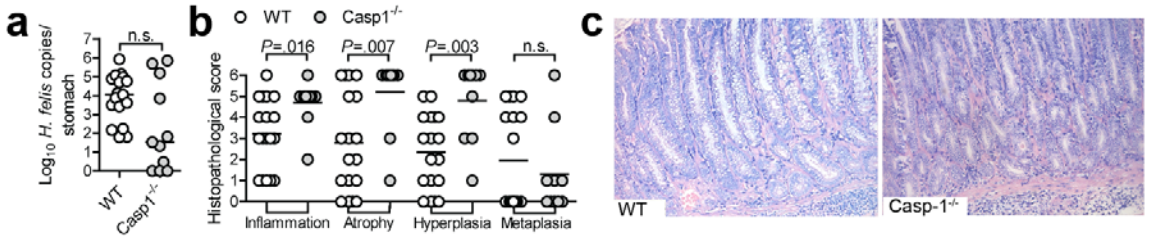
Supplemental Figures

Supplemental Figure 1



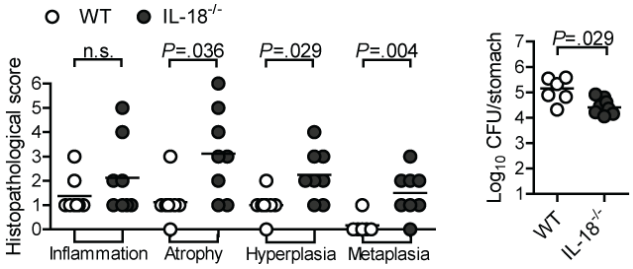
Suppl. Figure 1. Percentages with regard to total stomach cells of leucocytes ($CD45^+$) and $CD4^+$ T cells infiltrating the gastric mucosa in immunized & challenged (imm/inf) and infected (inf) wild type and caspase-$1^{-/-}$ mice; horizontal lines represent the medians.

Supplemental Figure 2



Suppl. Figure 2. (**a**) *H. felis* colonization as determined by quantitative PCR of the *flaB* gene; medians are represented by horizontal lines. (**b**) Histopathology scores of wild type and caspase-$1^{-/-}$ mice infected with *H. felis* for 3 months; horizontal lines indicate the means. (**c**) Representative micrographs of wild type and caspase-$1^{-/-}$ mice infected with *H. felis* for 3 months.

Supplemental Figure 3



Suppl. Figure 3. Histopathology scores of wild type and IL-18$^{-/-}$ mice infected with *H. pylori* PMSS1 for 1 month; horizontal lines indicate the means. *H. pylori* PMSS1 colonization levels as assessed by colony count assay; medians are represented by horizontal lines.

4. DISCUSSION

4.1 Requirements and obstacles of CFU-reducing anti-*H. pylori* immunity

Starting from the observation that some *Helicobacter*-infected C57BL/6 mice are able to control the bacterial loads better than their littermates, we set out to identify the factor(s) responsible for this difference. We noticed that mice which are able to clear the infection better are characterized by higher gastric expression of the cytokine IFN-γ. IFN-$\gamma^{-/-}$ mice exhibit higher bacterial burdens after experimental *H. pylori* infection than wild type mice and are less well protected by the gold standard oral vaccine regimen consisting of bacterial sonicate and CT. Therefore, we concluded that IFN-γ has a prominent role in the reduction of *Helicobacter* loads during spontaneous clearance as well as after challenge of vaccinated mice. Adoptive transfer of wild type, but not IFN-$\gamma^{-/-}$, $CD4^+$ T effector cells from an *H. felis*-infected donor to subsequently infected recipients lacking the recombination activating gene 1 (Rag-1; no mature T and B cells) or the T cell receptor beta chain (TCR-β; no α/β^+ T cells) induced complete bacterial clearance. Several additional immune cell types could be potential sources of IFN-γ. We could rule out a possible involvement of $CD8^+$ T lymphocytes. Adoptive transfer of high numbers of $CD8^+$ T cells could not mediate clearance in infected immunodeficient recipients. Moreover, $CD8^{-/-}$ mice did not manifest a defect in vaccination (unpublished observation). Depletion of natural killer (NK) and natural killer T (NKT) cells using PK136 antibody in experimentally infected mice or during the challenge phase of the vaccination protocol did lead to slight but not significant effects on bacterial colonization (data not shown). Similarly, the depletion of macrophages using liposomes loaded with chlodronate during challenge after vaccination did not impact the efficiency of vaccination (data not shown). In agreement with this finding, macrophages have been described as contributing to *H. pylori*-induced gastritis but not affecting bacterial loads.[177] Thus, IFN-γ-producing $CD4^+$ T helper cells proved to be crucial. Our results are in line with Akhiani et al. who find a role for Th1 cells after vaccination as well.[171] Nevertheless, the fact that IFN-$\gamma^{-/-}$ mice display a significant lack of protection in the CT vaccine model whereas MHC class II deficient mice, lacking functional $CD4^+$ T cells, are not protected at all but colonized like unvaccinated controls, suggested that there might be an additional $CD4^+$ T cell subset involved. It has been shown that IL-17 is induced in the *H. pylori*-infected human mucosa, that it is derived from

T cells and that IL-23 contributes to its induction.[178] In mice, lack of IL-17 leads to a significant reduction of neutrophil infiltration and myeloperoxidase (MPO) activity upon *H. pylori* infection compared to wild type mice.[179] Unexpectedly, however, IL-17$^{-/-}$ mice were found to be less colonized.[179] Recently, IL-17 levels have been shown to be increased after infection of animals vaccinated using CT[180] and two groups suggested IL-17 to be crucial for clearance since neutralizing IL-17 *in vivo* with a specific antibody significantly inhibited vaccine-induced protection.[181, 182] In contrast, *in vivo* neutralization of IFN-γ did not influence the outcome.[182] Moreover, a recent publication attributes the mucosal adjuvant effect of CT to Th17 responses.[183] Our data rather indicate that both cell types, Th1 and Th17, are required for reduction of *H. pylori* loads after CT vaccination, since in our hands animals lacking IFN-γ$^{-/-}$ do show a clear vaccination defect. We did not address the outcome of CT vaccination in absence of IL-17, but in line with Garhart et al.[172] we observed that mice lacking the common p40 subunit of IL-12 and IL-23, unable to mount Th1 and Th17 responses, are completely unprotected by our CT vaccine (unpublished data), which indeed suggests an involvement of both T cell populations. Using mice unable to mount either Th1 responses (IL-12p35$^{-/-}$), Th17 responses (IL-23p19$^{-/-}$), or both (IL-12/23p40$^{-/-}$), we could confirm the involvement of both T cell types in clearance after immunization with a new systemic adjuvant, CAF01 (see section 4.2). Analyzing the cytokine expression of CD4^{+} T cells infiltrating the stomach after challenge infection of mice vaccinated using CT or CAF01, we observed equally strong populations of mostly single positive IFN-γ- and IL-17-producing T cells. Only low numbers of TNF-α^{+} and no IL-5^{+} cells were detected. The discrepancies concerning the role of Th1 and/or Th17 responses in CFU-reducing immunity could indicate that both subsets may compensate for each other in certain settings. Clearance of bacteria after adoptive transfer of T effector cells into Rag-1$^{-/-}$ recipients for example is exclusively mediated by IFN-γ. On the other hand, animals lacking IL-18, generally known as a Th1 promoter,[184] are protected by vaccination in a manner comparable to wild type mice even though they display a clearly reduced IFN-γ response. Instead, this strain exhibits a significantly enhanced inherent IL-17 response and clearing capability (see section 4.4) which might make up for the lack of IFN-γ. However, another group has reported a lack of protection in IL-18$^{-/-}$ mice exactly because of a reduced Th1 response.[185] IL-1 receptor deficient mice cannot reduce bacterial loads after vaccination at all because they display severely impaired Th1 and Th17 responses, indicating a common need for IL-1 signaling (see section 4.4). Systemic vaccination using CagA instead of whole cell sonicate seemed to induce some bias to Th2-polarization given

the observed high levels of *H. pylori*-specific serum IgG1 (Th2 correlate) compared to IgG2c (Th1 correlate), which might be one reason why the immune response failed to clear the bacteria.

Among the potential downstream mediators of the T helper cells that mediate bacterial clearance, neutrophils and mast cells can be considered the most likely candidates. Neutrophils have been shown to be needed for efficient *H. pylori* clearance in IL-10$^{-/-}$ mice,[186] our adoptive transfer model of CD4^{+} effector T cells (from an infected donor) into infected Rag$^{-/-}$ mice as well as in a vaccine model.[180] Mast cells have been claimed to be crucial for the vaccine response because reconstitution of knockout mice (Kit^{W}/Kit^{W-v}) with mast cells was able to reestablish protection.[187] However, in a different model of mast cell deficiency (Kitl^{Sl}/Kitl^{Sl-d}) the effect was less drastic,[188] suggesting only a partial role for this cell type in reduction of bacterial loads. The concrete mechanism of bacterial elimination by help of mast cells remains elusive, since they are able to kill *H. pylori in vitro* but induction of gastric mast cell infiltration without vaccination does not lead to clearance.[187] While Velin et al. claim that neutrophil recruitment is not crucial for mast-cell mediated clearance in their hands,[187] Ding et al. suggest that mast cells are in crosstalk with CD4^{+} T cells and contribute to the recruitment of neutrophils.[188] We have analyzed gastric immune cell infiltration in our vaccine models and found increased numbers of mast cells and neutrophils after challenge. While CD4^{+} T cells infiltrated the mucosa at maximal numbers already on day 6 after infection, when the bacteria transiently had grown to high numbers, we could observe a correlation of the peak of neutrophil and mast cell infiltration with the most drastic change in *H. pylori* load between day 10 and day 14 after challenge (see Fig. 7).

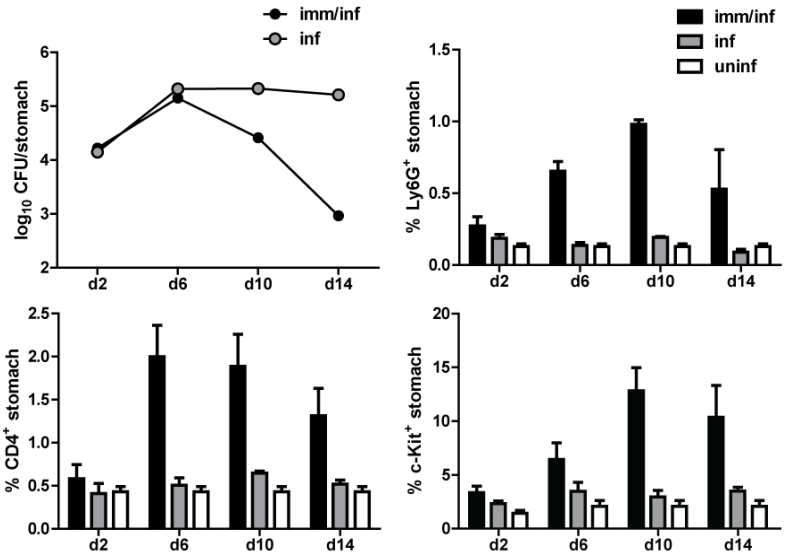


Fig. 7. Bacterial counts in CT-immunized compared to naive mice at 2, 6, 10 or 14 days after infection and stomach infiltration of CD4^{+} T cells, neutrophils (Ly6G^{+}) and mast cells (c-Kit^{+}) observed in these mice as well as uninfected controls.

However, the correlation of infiltrating mast cells with protection levels is less clear-cut than that of neutrophils. For instance, vaccination with recombinant CagA induced more pronounced infiltration of mast cells than of neutrophils and did not lead to protection. Neutrophils are known to phagocytose bacteria and indeed can partly be found close to *Helicobacter* in the gastric lumen (unpublished observations). Moreover, they seem to be required for efficient recruitment of $CD4^+$ T cells, and vice versa. Therefore, the main difference between infection and immunization seems to be the rapid recruitment of memory $CD4^+$ T helper cells and neutrophils to the gastric mucosa so that they can combat the bacteria before these are able to stably establish a high colonization level (see Fig. 7).

To our surprise we identified $CD11c^+$ DCs not to be required for efficient induction but, in contrast, to be a major obstacle of the anti-*H. pylori* vaccine response. Even partial depletion (about 50%) of $CD11c^+$ cells during challenge infection significantly improved the reduction of *H. pylori* counts. We have seen that contact of DCs with *H. pylori* renders them more tolerogenic than DCs from uninfected mice. Such DCs have an increased ability to induce regulatory $FoxP3^+$ T cells from naive $CD4^+CD25^-$ T cells in the presence of TGF-β *in vitro*. This is likely to happen also *in vivo* and to represent one major mechanism by which *H. pylori* induces immunological tolerance in order to establish chronic infection. Moreover, after challenge infection of immunized mice, such *H. pylori*-primed DCs may be able to convert Th17 memory cells generated by the preceding immunizations into Tregs. Support for this concept comes from a study where *in vitro H. pylori*-treated DCs induced a Th17-to-Treg shift when injected into subsequently infected mice.[189] Mechanistically, *Helicobacter* apparently induces a dominant semi-mature state of DCs, which are enriched in the infected mucosa,[189, 190] mostly positive for MHCII and expressing only low to moderate levels of the co-stimulatory molecules CD40, CD80, and CD86.[190] In a parallel experimental setup we depleted Tregs during challenge infection of CT- or CAF01-vaccinated mice, which, not surprisingly, strongly enhanced Th1 and Th17 responses and led to better clearance. Although the depletion of Tregs was more efficient (about 80%) than that of DCs, the observed effect was not much more potent, indicating that DCs are indeed crucial for induction of Tregs in this setting. Importantly, work from our lab has shown that also B cells can be implicated in *H. pylori*-driven Treg induction in that B cells activated by *H. pylori* via TLR2 generate IL-10 producing regulatory T cells of the Tr-1 type.[191] Having identified major causes currently hampering *H. pylori* vaccination efforts, the future focus can be put on potential strategies to circumvent these mechanisms. For instance, the

interaction between the co-stimulatory CD40 molecule on antigen presenting cells and CD40 ligand (CD40L) on T cells is important in the generation of a powerful adaptive immune response. Triggering of CD40 on DCs leads to activation, up-regulation of co-stimulatory and MHC molecules, enhanced production of IL-12, and efficient antigen presentation to T cells.[192] We have observed that it is possible to break immunological tolerance towards *H. pylori*, as it develops in mice that are neonatally infected,[67] by administering an agonistic anti-CD40 antibody during the first week of infection. We therefore thought it might be possible to improve vaccination by artificial CD40 triggering simultaneously with vaccination. However, if any, a converse effect was seen. Depleting Tregs during immunization using anti-CD25 antibody in our hands did not improve vaccination outcome either, although Zhang et al. have reported slight improvement of a suboptimal DC-based vaccine by Treg depletion.[147] Further efforts in this direction need to be undertaken.

4.2 A potential adjuvant candidate for a human *H. pylori* vaccine

Since CT is not an option for human use, and nontoxic versions of CT or alternatively LT administered orally could not convince in first human trials, we went on a quest for an alternative adjuvant. Since the 1920s, AlOH has been the only adjuvant approved for human use worldwide and it remains the only legal adjuvant in the US. In Europe, three additional adjuvants have been approved only recently: the squalene-based oil-in-water emulsions MF59 for influenza vaccination of the elderly (Novartis; 1997) and AS03 for a pandemic flu vaccine (GlaxoSmithKline (GSK); 2008), as well as AS04, containing the TLR4 agonist monophosphoryl lipid A (MPL) formulated in AlOH, for hepatitis B vaccination (GSK; 2005).[193] AlOH being the dominant human adjuvant with an irrevocable safety profile and having shown some rather promising preclinical results[132, 133] was a logical choice for the most recent human *H. pylori* vaccine trials initiated by Novartis.[155] However, AlOH is usually classified as Th2 adjuvant,[194, 195] and we like many others do not attribute CFU-reducing capacity to *H. pylori*-specific Th2 responses. We wondered if there was a better alternative given the requirements for successful *H. pylori* vaccination that we and others have observed, namely a mix of Th1 and Th17, or a very strong response of one of these types. CpG-ODN are known strong immune activators and especially inducers of Th1-biased responses. They were suggested to activate innate immunity

against already established *H. pylori* infection when administered alone via the oral route,[146] and have been tested several times as adjuvant in *H. pylori* vaccination. Giving CpG-ODN subcutaneously together with *H. pylori* lysate, however, only led to a 1-log-reduction of bacterial loads and at the same time to strongly increased gastritis.[135] Intranasal administration apparently has yielded miscellaneous results. While Shi et al. report eradication in about 80% of the immunized mice (assessed by rapid urease test),[130] Nyström et al. have only achieved three-fold reduction of bacteria.[196]

Since NK cells have been suggested to increase Th1 priming in mesenteric lymph nodes,[197] and we had observed slight, although not consistent, effects upon NK cell depletion in our vaccination model, we tested two adjuvants that reportedly recruit and activate NK cells, Ribi and R848.[197] The modes of action of these adjuvants are of course not restricted to NK cells. R848 is an imidazoquinoline compound that activates immune cells via the TLR7/TLR8 MyD88-dependent signaling pathway, triggers NF-κB activation and has potent anti-viral activity.[198, 199] Ribi no longer being available, we used its replacement Sigma Adjuvant System® (SAS), an oil-in-water emulsion containing MPL derived from *Salmonella enterica* and a synthetic analogue of trehalose dimycolate from mycobacterial cord factor in squalene and Tween 80. Like Ribi, it is intended as a less toxic alternative to CFA. In addition, since mast cells contribute to an efficient *H. pylori* vaccine response[187, 188] and have been described to mediate recruitment of T cells in draining lymph nodes[200] we have tested the mast cell activator c48/80 which has been attributed adjuvant function.[200, 201] Furthermore we have given the synthetic double-stranded RNA Poly(I:C) a try, which is a known TLR3 ligand and described as an efficient Th1-inducing adjuvant in a DC-targeted vaccine,[202] as well as the glycolipid α-galactosylceramide (αGalCer), a potent and specific activator of invariant NKT cells[203] which has successfully been used as adjuvant in various vaccines.[204-207] Finally, we have taken into consideration the adjuvant CAF01 because, even though it is very versatile and also can induce antibody-mediated protection, it elicits strong Th1 and Th17 responses in the context of mycobacterial vaccination.[208, 209] Its name stands for “cationic adjuvant formulation” and it consists of dimethyldioctadecylammonium (DDA) as liposome-forming delivery vehicle and trehalose-6,6-dibehenate (TDB), a synthetic mycobacterial cord factor, which is inserted into the lipid bilayers as immunomodulator. By means of the liposomes CAF01 targets antigens preferentially to antigen presenting cells, mainly dendritic cells, and leads to enhanced antigen uptake and presentation.[210, 211] Compared to AlOH, it elicits a strong early inflammatory response at the site of injection.[212] Among all the

mentioned adjuvant candidates we have tested, CAF01 stood out as the only one approaching the level of protection observed with CT (see Fig. 8). Combination of CAF01 with some of the other agents did not significantly improve its activity (data not shown).

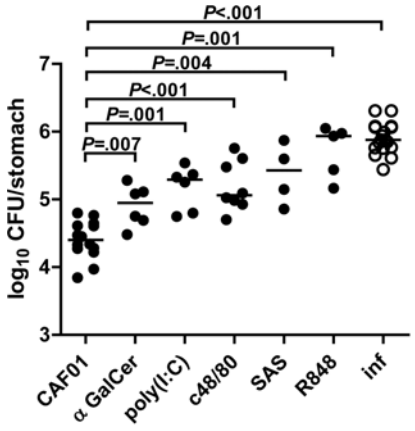


Fig. 8. *H. pylori* vaccination efficiency using various adjuvants. All agents were administered with 250 µg of *H. pylori* SS1 whole cell sonicate via the subcutaneous route, three times at weekly intervals; mice were challenged with SS1 two weeks after the last immunization.

Molecularly, the adjuvant action of CAF01 is based on Syk-Card9 signaling[209] and the FcRγ-associated C-type lectin receptor Mincle,[213] which distinguishes it from other adjuvants triggering TLRs (CpG, R848, poly(I:C), MPL) or the NALP3 inflammasome (AlOH).[214, 215] Since Card9 seems to be needed for the induction of Th17 responses to certain pathogens[216] this might explain CAF01's superiority over other adjuvants in vaccination against *M. tuberculosis* and *H. pylori*.

A major advantage of CAF01 is the fact that it has already been tested for safety and shown strong immunogenicity with a tuberculosis vaccine in two human trials (data not yet published).

In order to be a truly realistic human vaccine candidate it would of course be desirable to have a recombinant antigen cocktail rather than undefined bacterial sonicate administered with it. We have tested recombinant CagA as antigen given subcutaneously together with CAF01 and challenged the mice subsequently with the $CagA^+$ strain *H. pylori* PMSS1. We observed that despite a strong immune response towards the protein the bacterial counts were not affected after challenge compared to naive controls. Two early studies that have employed CagA as single antigen in prophylactic or therapeutic oral vaccination with detoxified LT had reported 75-80% protection.[122, 125] Both studies used the type I strain SPM326, however, which had been adapted to colonize the mouse stomach by repeated *in vivo* passages and thus might have lost its ability to deliver CagA. In systemic vaccines, CagA has only been used in conjunction with other antigens.[133, 155] CagA appears to be highly immunogenic but does not induce the required

protection (see also section 4.3). The composition of antigen mixtures intended for human use has to be scrutinized in this regard. Although the discovery of an adjuvant that is suitable for systemic administration of *H. pylori* antigens to humans and able to elicit the desired types of immune responses is encouraging, we are still far from achieving sterilizing immunity.

4.3 *H. pylori*-induced immunopathology and aggravation by non-sterile vaccination

As mentioned, we have observed significant variation in C57BL/6 mice concerning the outcome of *Helicobacter* infection. While most mice develop gastric preneoplastic lesions upon infection, some littermates remain unaffected. Th1 responses have been implicated in the development of gastritis before.[104, 217] In our hands, the level of gastric IFN-γ expression correlated closely with the grade of inflammation, gastric atrophy and hyperplasia, but not intestinal metaplasia, and mice deficient in IFN-γ developed significantly less pathology upon infection. There is no significant change of gastric gene expression in infected Rag-$1^{-/-}$ mice compared to uninfected wild type mice and adoptive transfer of $CD4^{+}$ T effector cells from infected wild type, but not IFN-$\gamma^{-/-}$ donors, is sufficient to trigger *Helicobacter*-associated preneoplasia. Moreover, pharmacological inhibition of *Helicobacter*-induced T effector cell responses prevents and even reverses already existing gastric lesions.[218, 219] All these findings suggest that the Th1-polarized immune response to *H. pylori* infection and the epithelial reaction to this immune response is the main underlying cause of gastric carcinogenesis. The genes we found to be up-regulated upon IFN-γ treatment in an immortalized primary gastric epithelial cell line (IMPGE) overlapped to a large degree with the genes whose expression correlated with gastric IFN-γ expression *in vivo*. Therefore it seems possible that IFN-γ has a direct influence on one or several lineages of gastric epithelial cells and triggers or contributes to their progression to a preneoplastic phenotype, especially hyperplasia. In contrast, metaplasia may be driven by a different pathway. Given the recently identified role of IL-17 responses in mediating inflammation and clearance upon *H. pylori* infection, it will be interesting to analyze possible direct effects of IL-17 on gastric epithelial cell lineages and to dissect the respective contributions of Th1 and Th17 responses to *H. pylori*-induced immunopathology. The latter will be achieved by analyzing the outcome of long-term infection of IL-12p$35^{-/-}$, IL-23p$19^{-/-}$ and IL-12/23p$40^{-/-}$ mice, which cannot mount Th1, Th17 or both responses, compared to wild type. The observation that *Helicobacter*-infected

caspase-$1^{-/-}$ and IL-$18^{-/-}$ mice show enhanced IL-17 levels and gastric pathology compared to wild type (see section 4.4) already hints at a possible pathogenic potential of Th17 responses in gastric inflammation.

The fact that oral or systemic immunization using CagA as antigen induces an excessive immune response but no obvious change in bacterial burden sheds new light on the carcinogenic properties of this protein. It seems to be a highly immunogenic, but not a protective antigen. Besides its direct effects on epithelial and immune cells it can therefore contribute to gastric pathology by its strong T cell antigenicity. This possibly even represents a major mechanism of CagA-induced preneoplastic changes since mice that have developed tolerance to *H. pylori* are protected from pathology even when colonized with high numbers of $CagA^+$ bacteria.[67]

We and others have shown that the Th1 responses causing gastric preneoplasia are at the same time required for reducing bacterial loads, [171, 220] be it after infection, adoptive transfer or vaccination, as discussed above. This is unproblematic in case the infection gets eradicated completely because gastritis can resolve. However, under normal circumstances *H. pylori* is not cleared from the stomach. The goal of vaccination is of course to achieve this, but so far most vaccines fail to do so and leave some residual colonization. Knowledge on what happens after incomplete eradication of *H. pylori* after immunization is not exhaustive. Our results definitely suggest that vaccination increases epithelial damage caused by *H. pylori* infection as long as it does not achieve sterilizing immunity. We first observed a significant increase in gastric infiltration and pathological parameters when comparing immunized animals seven weeks after challenge infection to non-immunized controls. This observed increase in inflammation and pathology formation was even more drastic when comparing groups twelve weeks after challenge infection. In accordance with these findings for *H. pylori* SS1, we noticed a strong increase in pathology in mice immunized against and subsequently infected with *H. pylori* PMSS1 eight weeks after infection compared to unimmunized controls. In all cases, the vaccinated mice were able to significantly and stably reduce the bacteria but not to entirely clear the infection. These data clearly show that vaccination and subsequent challenge infection cause a lingering chronic inflammation which is worse than after infection alone, unless the bacteria triggering the immune response are completely eliminated. There is no reason to assume that these effects would subside at later time points except the residual bacteria are finally cleared. However, previous work by Sutton et al. suggested that even after non-sterile immunization the so-called “postimmunization gastritis” after challenge infection with *H. felis*

was transient, went down to levels of naive mice after six months and therefore did not exacerbate pathology.[221] A possible explanation for this discrepancy would be the later time point and/or the different strains studied, namely *H. felis* in the case of Sutton et al. and *H. pylori* in our case. But our data are in line with findings from Ermak et al. who used *H. felis* as challenge strain as well and found epithelial alterations in 20% of the immunized mice after one year, which could be alleviated by antibiotic treatment, suggesting that they were due to residual bacteria.[124] Gastritis has also been observed after therapeutic SS1 immunization in protected but not unprotected mice.[127, 222] Michetti et al. have reported earlier that a residual low-level infection after *H. felis* challenge remained in a fraction of immunized mice, which got eliminated over time, however.[129] Similarly, Garhart et al. observed a peak of inflammation in immunized mice one to two weeks post challenge and a gradual decrease to normal levels over the course of one year with concomitant clearance of SS1 infection.[223] Likewise, gastritis peaked nine weeks after adoptive transfer of splenocytes into SS1-infected severe combined immunodeficient (SCID) mice and had resolved after 45 weeks together with complete elimination of the bacteria.[224] In our studies we did not analyze the outcome of vaccination later than three months after infection, and until then the bacterial colonization had remained stable at low levels. Possibly we would have been able to observe complete clearance at least in some animals at later points in time, but in any case such a protracted effect is not acceptable for a vaccine which should act in a more immediate manner. This seems to be the case in Mongolian gerbils. Six weeks after *Helicobacter* challenge, 80% of the orally immunized animals cleared the infection and no postimmunization gastritis was detectable.[225] Unfortunately, most of the numerous publications on *Helicobacter* vaccination do not address the issue of post-immunization/challenge pathology because of the required long-term follow-up. The available data, however, underline that the often invoked “protective immunity” elicited by vaccination against *H. pylori* can at the same time be a major cause of gastric immunopathology. Since *H. pylori*-induced immunity seems to have a lot more importance in induction of preneoplastic lesions than any direct effects of the bacteria on host cells, the term “protective” is somewhat misleading. Every *H. pylori* vaccine that fails to confer sterile immunity is likely to increase rather than prevent the vaccinee’s risk of developing gastric pathology.

If vaccine development should stagnate at one point due to insolvable issues, active tolerization against *H. pylori* might represent an alternative strategy. We could show that administration of an antibody blocking the CD40-CD40L interaction together with whole cell sonicate of *H. pylori*

PMSS1 during the neonatal period protected mice from developing inflammation and immunopathology when they were infected as adults. At the same time, they displayed increased bacterial colonization. Similar, though weaker effects were observed when administering CagA instead of sonicate along with CD40L-neutralizing antibody. Tolerization strategies of that kind are not yet established for human use but might be worthwhile exploring in the future.

4.4 A regulatory function of the inflammasome in *H. pylori* infection

Having observed reduced efficacy of *H. pylori* vaccination in caspase-1 deficient mice prompted us to investigate in more detail the significance of this finding, i.e. if and how caspase-1 is involved in detection of *H. pylori* and the possible implications on the outcome of infection.

We could show that MLN from infected animals contain significantly more cells that display caspase-1 activation than uninfected controls, and confirm *in vitro* that *H. pylori* bacteria are able to activate caspase-1 in a substantial fraction of BMDCs. This process is contact-dependent, but CagA-independent, because caspase-1 activation is abrogated when the bacteria are separated from the cells in a transwell chamber, however not when a CagE-deficient strain is used (data not shown). The NLR required upstream of inflammasome activation by *H. pylori* is currently unknown. Since the main function of caspase-1 is the processing of the immature forms of the cytokines IL-1 and IL-18, we confirmed caspase-1 activation by measuring increased secretion of IL-1β and IL-18 from infected BMDCs and gastric mucosa. These observations and the reduced protection in the vaccine model in the absence of caspase-1 hinted at a role of inflammasome signaling in promoting *H. pylori*-induced inflammation and immune activation. On the other hand, naive caspase-$1^{-/-}$ mice displayed a better spontaneous clearance and stronger Th17 responses compared to infected wild type mice. Moreover, much to our surprise, caspase-$1^{-/-}$ mice developed more severe gastric lesions after *H. felis* infection, suggesting that caspase-1 is not only able to enhance but also to downregulate *H. pylori*-specific immunity and immunopathology. We subsequently analyzed mice lacking IL-18 or the receptor for IL-1 (IL-1R) in order to possibly attribute the complex effects observed with caspase-1-deficient mice to these two downstream mediators.

In terms of vaccination, it can be conceived that lack of caspase-1 leads to a significant defect due to reduced processing of IL-1β. The observed complete lack of protection in IL-1R$^{-/-}$ mice, which have severely impaired Th1 as well as Th17 responses, ascertains that IL-1 signaling is strictly required for vaccine efficacy. It might be that no such drastic effect is seen in caspase-1$^{-/-}$ because mature IL-1β is still available from other, inflammasome-independent sources such as activated neutrophils.[226] The fact that caspase-1$^{-/-}$ mice have unimpaired or even enhanced Th17 responses actually supports this hypothesis since IL-1β is required for Th17 development.[227] In contrast, lack of IL-18 does not hamper vaccination. This finding was initially astonishing as IL-18 has been identified as "interferon-gamma inducing factor"[184] and is generally described as the cytokine driving Th1 responses,[228] besides IL-12. As alluded to above, the impaired Th1 response in this background might be made up for by inherently increased IL-17 levels that are seen in infected IL-18$^{-/-}$ mice similarly to caspase-1$^{-/-}$ mice.

In terms of *Helicobacter*-induced pathology, IL-1R$^{-/-}$ mice are completely protected from pathology development, underlining the importance of IL-1 signaling in driving *Helicobacter*-associated disease. In contrast, IL-18$^{-/-}$ mice closely mimic the caspase-1$^{-/-}$ phenotype and display enhanced gastric pathology. Since Th1 responses are an important driving force of gastric preneoplasia, IL-18$^{-/-}$ mice could have been expected to be somewhat protected. Thus, our observations propose a different, regulatory function for IL-18 in our setting. In models of intestinal inflammation, IL-18 has already been reported to be beneficial,[229, 230] with the mechanism still being unclear. It has been suggested that IL-18 is contributing to maintenance of the epithelial barrier function in response to danger signals from damaged tissue.[231] On the other hand it has also been claimed that caspase-1 activity and IL-18 secretion from immune cells drive inflammation and tumorigenesis in the colon.[232, 233] Overall, it seems that IL-18 is involved in various mechanisms and its role varies depending on the model and the time point analyzed.

Our data favor a predominant role of IL-18 signaling from and to the immune cell compartment. Several preliminary observations support this idea. Bone marrow transfer experiments showed that wild type recipients of caspase-1$^{-/-}$ bone marrow cells developed pathology as observed in the caspase-1$^{-/-}$ background. Apart from that, we see slightly reduced Foxp3^{+}CD4^{+} T cell fractions in the stomach infiltrate as well as in MLN of infected IL-18$^{-/-}$ compared to wild type mice, albeit the differences are not significant. Moreover, naive CD4^{+} T effector cells lacking the receptor for IL-18 convert to a lesser extent to FoxP3^{+}CD25^{+} Tregs than wild type cells in culture with

H. pylori-pre-treated DCs and TGF-β (see Fig. 9A). ELISA results on co-culture supernatants indicate that IL-18R$^{-/-}$ T cells might instead convert more to Th17 cells. Interestingly however, their CD25 expression does not seem to differ much from converted wild type T cells.

We have also started to examine the role of IL-18 in immune regulation in other *in vivo* models. As referred to above, mice infected with *H. pylori* at a neonatal age develop tolerance and, though highly colonized, are protected from immunopathology throughout adulthood.[67] Our group has recently shown that CD4$^+$CD25$^+$ Tregs isolated from mesenteric lymph nodes of such tolerant mice have a protective effect upon adoptive transfer into an experimental asthma model (Arnold et al., accepted manuscript). Allergic airway disease is induced by ovalbumin sensitization and subsequent aerosol challenge in this model and characterized by airway hyperresponsiveness, tissue inflammation and goblet cell metaplasia. Another hallmark is the pulmonary and bronchoalveolar infiltration of Th2 and Th17 cells as well as eosinophils, and cell counts in the bronchoalveolar lavage fluid (BALF) serve as a reliable disease correlate, alongside measurements of respiratory resistance to inhaled methacholine. Infection with *H. pylori* prior to asthma induction, especially during the neonatal period, protects mice from these symptoms. This protection can be transferred to uninfected recipients by MLN Tregs and is associated with an immature DC phenotype and Treg accumulation in the lung.

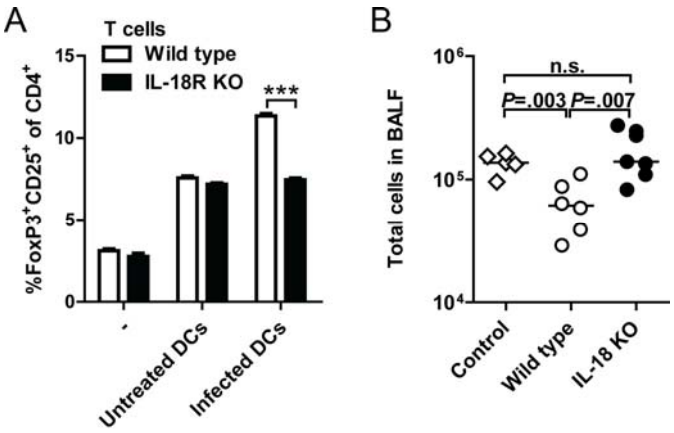


Fig. 9. A) Conversion of wild type and IL-18R$^{-/-}$ CD4$^+$CD25$^-$ T cells into FoxP3$^+$CD25$^+$ Tregs cultured alone or in co-culture with untreated or *H. pylori*-pre-treated BMDCs.
B) Total cell counts in BALF from OVA-sensitized recipients of MLN CD4$^+$CD25$^+$ T cells isolated from neonatally infected wild type or IL-18$^{-/-}$ mice compared to positive controls after OVA challenge.

We wondered if IL-18-deficient mice develop tolerance towards *H. pylori* upon neonatal infection as wild type mice. In addition, in order to test their *in vivo* functionality, we wanted to see how cells from neonatally infected IL-18$^{-/-}$ mice perform compared to wild type cells in terms of asthma protection. First results suggest that IL-18$^{-/-}$ mice do not develop full tolerance upon neonatal infection. Moreover, CD4$^+$CD25$^+$ T cells isolated from these mice do not confer

asthma protection since recipients develop asthma-like symptoms comparably to mice that did not receive any cells (see Fig. 9B). This could imply a functional defect of IL-18$^{-/-}$ Tregs or be due to a lower number of Foxp3^{+} cells in the CD4^{+}CD25^{+} T cells isolated from IL-18$^{-/-}$ mice, which would match with our other observations and will be verified in upcoming experiments. With the apparent bias towards IL-17 production, these cells might actually make things slightly worse.

Our findings have to be confirmed and expanded, for example by looking at the possibility to induce Treg skewing by recombinant IL-18 *in vitro* and/or *in vivo*. At the moment we can only speculate how exactly IL-18 exerts regulatory function. IL-18 secretion by tolerogenic DCs might be required for Treg induction or regulation of the Th17-Treg balance. In fact, TGF-β, which is known to inhibit DC maturation and to render them tolerogenic, especially at mucosal sites,[234] has been shown to induce IL-18 expression in DCs, whereas it down-regulates IL-12.[235] The development of Tregs and Th17 cells has been demonstrated to be closely linked, for instance, they both require TGF-β,[236] and FoxP3^{+}IL-17^{+} double positive cells can be observed early in the differentiation process.[237] Moreover, once established, Tregs have been described to induce rather than suppress Th17 responses.[236, 238, 239] They potentially promote Th17 cells by secretion of TGF-β, induction of IL-10 in DCs, generally by inhibiting Th1 and Th2 responses, which is favorable for Th17 differentiation,[236] or regulation of IL-2 availability.[239] Furthermore, the cell lineages retain plasticity, in particular, Tregs can convert to Th17 cells under the influence of IL-1β and IL-6 *in vitro* and *in vivo*.[237, 240, 241] IL-18 might directly, by binding to its receptor on T cells, or indirectly, via its strong potential to induce IFN-γ production,[113, 242] inhibit Th17 development and thereby favor generation of Tregs. Thus it could be that IL-18 secretion elicited by *H. pylori* infection ensures a "normal" Treg-to-Th17 ratio, whereas in the absence of IL-18 Th17 cells prevail since an important blocking factor is gone and IL-1β together with other cytokines promotes their differentiation. In turn, this might lead to slightly reduced Treg numbers since development of Th17 cells is favored over that of Tregs from common precursors. Another possibility would be that IL-18 directly promotes Treg development or converts already established Th17 cells to Tregs. In either case, Th17 responses have also been reported to be increased in an atherosclerosis model in IL-18-deficient mice.[243]

Taken together, it seems that the sensing of *Helicobacter* by the inflammasome does not contribute as much to elimination of infection and immunopathology, potentially because of IL-1β from other sources, as to protection against infection-induced damage by limiting

excessive Th17 cell responses. Therefore it may represent yet another mechanism by which *Helicobacter* ensures its persistence in the host (see Fig. 10).

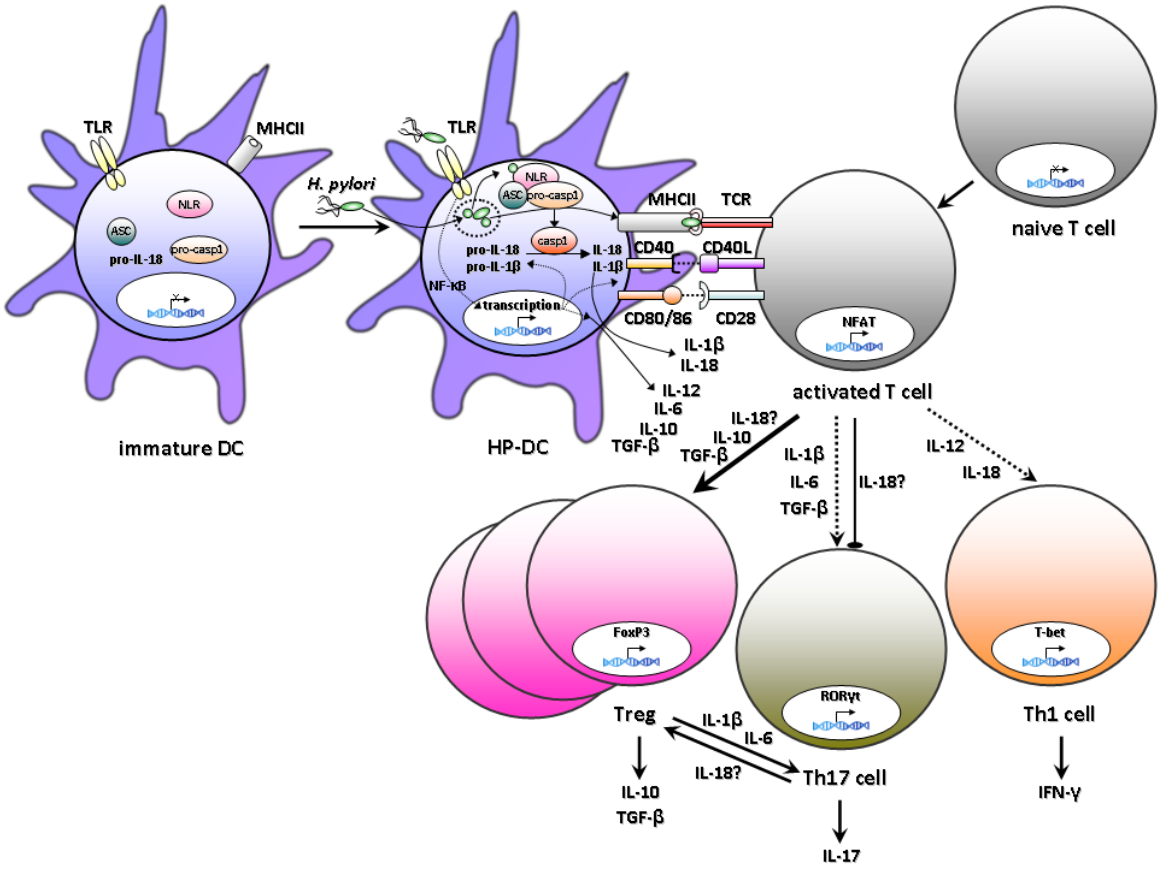


Fig. 10. Schematic of the mechanisms leading to preferential Treg induction by *H. pylori*-experienced DCs (HP-DC). Contact with *H. pylori* induces a semi-mature state of DCs, i.e. they present *H. pylori* antigens on MHCII but only low to moderate levels of co-stimulatory molecules on their surface, which per se favors the induction of Tregs. As a consequence of rather weak TLR triggering by *H. pylori* and subsequent signaling via NF-κB and other pathways not depicted here, several cytokines with pro-inflammatory and tolerogenic properties are expressed and secreted. In addition, inflammasome activation by *H. pylori* leads to processing and secretion of IL-1β and IL-18. According to our data, IL-18 promotes Th1 cells but on the other hand also either directly induces Tregs, inhibits Th17 development or induces Th17-to-Treg skewing. The overall net result is the induction of a limited amount of Th1 and Th17 cells while Tregs prevail.

5. CONCLUSIONS AND PERSPECTIVES

There has been a long-standing interest in developing a vaccine against *H. pylori* almost ever since the discovery of its implication in gastric disease. Due to the increasing antibiotic resistance of *H. pylori* strains and therefore decreasing efficacy of *H. pylori* eradication therapy in clinical practice[37] this interest is renewed nowadays. Recent analyses for the United States and China suggest that preventing infection by vaccination in childhood could be a cost-effective measure able to reduce gastric cancer incidence by more than 40%.[244, 245] Slow but steady progress has been made in *H. pylori* vaccine development. Besides new approaches to mucosal immunization, for instance via the sublingual[246] or pulmonary route,[247] the feasibility of systemic immunization has been demonstrated. Potential human-use adjuvants such as CAF01 have come into play. A list of recombinant antigens has been identified as promising vaccine candidates, even though a question mark has to be put to the suitability of CagA. Our and the work of others now provide convincing evidence that a local $CD4^+$ T cell-mediated response of the Th1 and Th17 types is required for reduction of *H. pylori* loads. In addition, the mechanisms that prevent complete eradication of *H. pylori* are better understood. While IFN-γ- and IL-17-secreting T helper cells are certainly also induced by *H. pylori* infection, they are suppressed by Tregs that are preferentially induced by *H. pylori*-experienced DCs and favor bacterial persistence. Vaccination elicits *H. pylori*-specific memory $CD4^+$ T cells that can alter the ratio between T helper and Treg responses to such an extent that the bacteria are significantly reduced. The only way to achieve sterile immunity seems to be to overcome *H. pylori*-mediated immune regulation more efficiently. This has to be achieved already before *H. pylori* actually comes into play, of course, at the time of vaccination by inducing even stronger memory responses. The novel insights we have gained on the role of innate immune signaling via the inflammasome in the context of *H. pylori* infection might provide new avenues for improvement of vaccination. The induction of IL-18 via caspase-1 activation in DCs possibly represents a further crucial mechanism by which *H. pylori* prevents the host's immune response from eradicating it. Once fully elucidated, it could be envisaged to exploit this mechanism for a truly effective vaccine regimen. Apart from that, it will be worthwhile to explore neonatal immunization strategies since a human vaccine would be most likely aimed for this age group, which implies particular requirements. Unfortunately, all currently thinkable human vaccine regimens would possibly increase rather than prevent gastric pathology. Moreover, concerns are raised concerning the

presently observed epidemic increase of esophageal disease, ranging from gastroesophageal reflux disease to esophageal adenocarcinoma, coinciding with the rapid decline of *H. pylori* infections in the Western world. An inverse correlation of *H. pylori* infection and associated disease with Barrett's esophagus has been reported several times.[248] Further observations, implying an inverse correlation of asthma and allergies[249, 250] as well as obesity[251, 252] with *H. pylori* infection, give rise to skepticism towards a complete eradication of this almost symbiotic human pathogen. On the other hand, there are also extragastric diseases where *H. pylori* eradication is beneficial, such as iron deficiency anemia[253, 254] and idiopathic immune thrombocytopenia.[255-257] Generally, assumptions of beneficial or harmful associations of *H. pylori* infection have recently increased somewhat disproportionally to their scientific proof. In conclusion, in parallel to the search for a sterilizing vaccine, all consequences of *H. pylori* eradication or persistence must be taken into account when deliberating about the most valid future strategy of dealing with this infection.

6. REFERENCES

1. O'Rourke, J. and G. Bode, Chapter 6: Morphology and ultrastructure. Helicobacter pylori: physiology and genetics, ed. H. Mobley, G. Mendz, and S. Hazell. 2001, Washington (DC): ASM Press.
2. Euzéby, J.P. List of prokaryotic names with standing in nomenclature - genus Helicobacter [cited 2011 March 4]; Available from: http://www.bacterio.cict.fr/h/helicobacter.html.
3. Mikkonen, T.P., R.I. Karenlampi, and M.-L. Hanninen, Phylogenetic analysis of gastric and enterohepatic Helicobacter species based on partial HSP60 gene sequences. Int J Syst Evol Microbiol, 2004. 54(3): p. 753-758.
4. Mobley, H., Chapter 16: Urease. Helicobacter pylori: physiology and genetics, ed. H. Mobley, G. Mendz, and S. Hazell. 2001, Washington (DC): ASM Press.
5. Andrutis, K., et al., Inability of an isogenic urease-negative mutant stain of Helicobacter mustelae to colonize the ferret stomach. Infect Immun, 1995. 63(9): p. 3722-3725.
6. Eaton, K.A., et al., Essential role of urease in pathogenesis of gastritis induced by Helicobacter pylori in gnotobiotic piglets. Infect Immun, 1991. 59(7): p. 2470-2475.
7. O'Toole, P.W., M.C. Lane, and S. Porwollik, Helicobacter pylori motility. Microb Infect, 2000. 2(10): p. 1207-1214.
8. Fox, J.G. and T.C. Wang, Inflammation, atrophy, and gastric cancer. J Clin Invest, 2007. 117(1): p. 60-69.
9. O'Keeffe, J. and A.P. Moran, Conventional, Regulatory, and Unconventional T Cells in the Immunologic Response to Helicobacter pylori. Helicobacter, 2008. 13(1): p. 1-19.
10. Kusters, J.G., A.H.M. van Vliet, and E.J. Kuipers, Pathogenesis of Helicobacter pylori Infection. Clin Microbiol Rev, 2006. 19(3): p. 449-490.
11. Odenbreit, S., et al., Translocation of Helicobacter pylori CagA into gastric epithelial cells by type IV secretion. Science, 2000. 287(5457): p. 1497-500.
12. Stein, M., R. Rappuoli, and A. Covacci, Tyrosine phosphorylation of the Helicobacter pylori CagA antigen after cag-driven host cell translocation. Proc Natl Acad Sci U S A, 2000. 97(3): p. 1263-8.
13. Gebert, B., et al., Helicobacter pylori vacuolating cytotoxin inhibits T lymphocyte activation. Science, 2003. 301(5636): p. 1099-102.
14. Cover, T.L. and S.R. Blanke, Helicobacter pylori VacA, a paradigm for toxin multifunctionality. Nat Rev Micro, 2005. 3(4): p. 320-332.
15. Carlsohn, E., et al., HpaA is essential for Helicobacter pylori colonization in mice. Infect Immun, 2006. 74(2): p. 920-926.
16. Wang, G., et al., Dual roles of Helicobacter pylori NapA in inducing and combating oxidative stress. Infect Immun, 2006. 74(12): p. 6839-6846.
17. Satin, B., et al., The neutrophil-activating protein (Hp-Nap) of Helicobacter pylori is a protective antigen and a major virulence factor. J Exp Med, 2000. 191(9): p. 1467-1476.
18. Stein, M., et al., c-Src/Lyn kinases activate Helicobacter pylori CagA through tyrosine phosphorylation of the EPIYA motifs. Mol Microbiol, 2002. 43(4): p. 971-80.
19. Suerbaum, S. and C. Josenhans, Helicobacter pylori evolution and phenotypic diversification in a changing host. Nat Rev Micro, 2007. 5(6): p. 441-452.
20. Suerbaum, S. and P. Michetti, Helicobacter pylori infection. N Engl J Med, 2002. 347(15): p. 1175-1186.
21. Lee, Y., et al., Eradication of Helicobacter pylori to prevent gastroduodenal diseases: Hitting more than one bird with the same stone. Therap Adv Gastroenterol, 2008. 1(2): p. 111-120.
22. Granstrom, M., Y. Tindberg, and M. Blennow, Seroepidemiology of Helicobacter pylori infection in a cohort of children monitored from 6 months to 11 years of age. J Clin Microbiol, 1997. 35(2): p. 468-470.
23. Rothenbacher, D., et al., Acquisition of Helicobacter pylori infection in a high-risk population occurs within the first 2 years of life. J Pediatr, 2000. 136(6): p. 744-748.

24. Goodman, K.J., et al., Dynamics of Helicobacter pylori infection in a US–Mexico cohort during the first two years of life. Int J Epidemiol, 2005. 34(6): p. 1348-1355.
25. Perez-Perez, G.I., et al., Transient and persistent Helicobacter pylori colonization in native American children. J Clin Microbiol, 2003. 41(6): p. 2401-2407.
26. Magalhães Queiroz, D.M. and F. Luzza, Epidemiology of Helicobacter pylori Infection. Helicobacter, 2006. 11: p. 1-5.
27. Brown, L.M., Helicobacter pylori: Epidemiology and routes of transmission. Epidemiol Rev, 2000. 22(2): p. 283-297.
28. Ernst, P.B. and B.D. Gold, The disease spectrum of Helicobacter pylori: The immunopathogenesis of gastroduodenal ulcer and gastric cancer. Annu Rev Microbiol, 2000. 54(1): p. 615-640.
29. Marshall, B.J. and J.R. Warren, Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet, 1984. 1(8390): p. 1311-5.
30. Marshall BJ, et al., Attempt to fulfil Koch's postulates for pyloric Campylobacter. Med J Aust, 1985. 142(8): p. 436-9.
31. Peek, R.M., Jr. and M.J. Blaser, Helicobacter pylori and gastrointestinal tract adenocarcinomas. Nat Rev Cancer, 2002. 2(1): p. 28-37.
32. Parsonnet, J., et al., Helicobacter pylori infection and the risk of gastric carcinoma. N Engl J Med, 1991. 325(16): p. 1127-31.
33. Parsonnet, J., et al., Helicobacter pylori infection and gastric lymphoma. N Engl J Med, 1994. 330(18): p. 1267-71.
34. WHO, Infection with Helicobacter pylori. Schistosomes, liver flukes and Helicobacter pylori, 1994. 61: p. 177-241.
35. WHO. Fact sheet n°297: Cancer [cited 2011 March 1]; Available from: http://www.who.int/mediacentre/factsheets/fs297/en/index.html.
36. Ford, A.C., et al., Eradication therapy for peptic ulcer disease in Helicobacter pylori positive patients. CDSR, 2006(2): p. CD003840.
37. Graham, D.Y. and L. Fischbach, Helicobacter pylori treatment in the era of increasing antibiotic resistance. Gut, 2010. 59(8): p. 1143-1153.
38. Parsonnet, J., What is the Helicobacter pylori global reinfection rate? Can J Gastroenterol, 2003. 17 Suppl B: p. 46B-48B.
39. Gisbert, J.P., The recurrence of Helicobacter pylori infection: Incidence and variables influencing it. A critical review. Am J Gastroenterol, 2005. 100(9): p. 2083-2099.
40. Take, S., et al., The long-term risk of gastric cancer after the successful eradication of Helicobacter pylori. J Gastroenterol, 2010: p. 1-7.
41. Uemura, N., et al., Effect of Helicobacter pylori eradication on subsequent development of cancer after endoscopic resection of early gastric cancer. Cancer Epidemiol Biomarkers Prev, 1997. 6(8): p. 639-642.
42. Kato, M., et al., Eradication of Helicobacter pylori for primary gastric cancer and secondary gastric cancer after endoscopic mucosal resection. J Gastroenterol, 2007. 42(0): p. 16-20.
43. Parsonnet, J., et al., Should we treat H. pylori infection to prevent gastric cancer? Gastroenterology, 1997. 112(3).
44. Wong, B.C.-Y., et al., Helicobacter pylori eradication to prevent gastric cancer in a high-risk region of China. JAMA, 2004. 291(2): p. 187-194.
45. You, W.-c., et al., Randomized double-blind factorial trial of three treatments to reduce the prevalence of precancerous gastric lesions. JNCI, 2006. 98(14): p. 974-983.
46. Ford, A.C. and A.T.R. Axon, Epidemiology of Helicobacter pylori infection and public health implications. Helicobacter, 2010. 15: p. 1-6.
47. Correa, P. and J. Houghton, Carcinogenesis of Helicobacter pylori. Gastroenterology, 2007. 133(2): p. 659-672.
48. Parsonnet, J., et al., Risk for gastric cancer in people with CagA positive or CagA negative Helicobacter pylori infection. Gut, 1997. 40(3): p. 297-301.

49. Rieder, G., J.L. Merchant, and R. Haas, Helicobacter pylori cag-type IV secretion system facilitates corpus colonization to induce precancerous conditions in Mongolian gerbils. Gastroenterology, 2005. 128(5): p. 1229-42.
50. Hatakeyama, M., Oncogenic mechanisms of the Helicobacter pylori CagA protein. Nat Rev Cancer, 2004. 4(9): p. 688-694.
51. Saadat, I., et al., Helicobacter pylori CagA targets PAR1/MARK kinase to disrupt epithelial cell polarity. Nature, 2007. 447(7142): p. 330-333.
52. Murata-Kamiya, N., et al., Helicobacter pylori CagA interacts with E-cadherin and deregulates the [beta]-catenin signal that promotes intestinal transdifferentiation in gastric epithelial cells. Oncogene, 2007. 26(32): p. 4617-4626.
53. Ohnishi, N., et al., Transgenic expression of Helicobacter pylori CagA induces gastrointestinal and hematopoietic neoplasms in mouse. Proc Natl Acad Sci U S A, 2008. 105(3): p. 1003-1008.
54. Fox, J.G., et al., Host and microbial constituents influence Helicobacter pylori-induced cancer in a murine model of hypergastrinemia. Gastroenterology, 2003. 124(7): p. 1879-90.
55. Fox, J.G., et al., Helicobacter pylori-associated gastric cancer in INS-GAS mice is gender specific. Cancer Res, 2003. 63(5): p. 942-50.
56. Fox, J.G., et al., Concurrent enteric helminth infection modulates inflammation and gastric immune responses and reduces helicobacter-induced gastric atrophy. Nat Med, 2000. 6(5): p. 536-42.
57. El-Omar, E.M., et al., Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature, 2000. 404(6776): p. 398-402.
58. Tu, S., et al., Overexpression of Interleukin-1 beta Induces Gastric Inflammation and Cancer and Mobilizes Myeloid-Derived Suppressor Cells in Mice. Cancer Cell, 2008. 14(5): p. 408-419.
59. El-Omar, E.M., et al., Increased risk of noncardia gastric cancer associated with proinflammatory cytokine gene polymorphisms. Gastroenterology, 2003. 124(5): p. 1193-1201.
60. Thye, T., et al., Genomewide linkage analysis identifies polymorphism in the human interferon-gamma receptor affecting Helicobacter pylori infection. Am J Hum Genet, 2003. 72(2): p. 448-53.
61. Canedo, P., et al., The interferon gamma receptor 1 (*IFNGR1*) -56C/T gene polymorphism is associated with increased risk of early gastric carcinoma. Gut, 2008. 57(11): p. 1504-1508.
62. Karita, M., et al., New small animal model for human gastric Helicobacter pylori infection: success in both nude and euthymic mice. Am J Gastroenterol, 1991. 86(11): p. 1596-603.
63. Lee, A., et al., A small animal model of human Helicobacter pylori active chronic gastritis. Gastroenterology, 1990. 99(5): p. 1315-23.
64. Marchetti, M., et al., Development of a mouse model of Helicobacter pylori infection that mimics human disease. Science, 1995. 267(5204): p. 1655-8.
65. Lee, A., et al., A standardized mouse model of Helicobacter pylori infection: introducing the Sydney strain. Gastroenterology, 1997. 112: p. 1386–1397.
66. Crabtree, J.E., R.L. Ferrero, and J.G. Kusters, The mouse colonizing Helicobacter pylori strain SS1 may lack a functional cag pathogenicity island. Helicobacter, 2002. 7(2): p. 139-140.
67. Arnold, I.C., et al., Tolerance rather than immunity protects from Helicobacter pylori-induced gastric preneoplasia. Gastroenterology, 2011. 140(1): p. 199-209.e8.
68. Fox, J.G., et al., Germ-line p53-targeted disruption inhibits helicobacter-induced premalignant lesions and invasive gastric carcinoma through down-regulation of Th1 proinflammatory responses. Cancer Res, 2002. 62(3): p. 696-702.
69. Rogers, A.B. and J.G. Fox, Inflammation and cancer I. Rodent models of infectious gastrointestinal and liver cancer. Am J Physiol Gastrointest Liver Physiol, 2004. 286(3): p. G361-G366.
70. Enno, A., et al., MALToma-like lesions in the murine gastric mucosa after long-term infection with Helicobacter felis. A mouse model of Helicobacter pylori-induced gastric lymphoma. Am J Pathol, 1995. 147(1): p. 217-22.
71. Mueller, A., et al., The role of antigenic drive and tumor-infiltrating accessory cells in the pathogenesis of helicobacter-induced mucosa-associated lymphoid tissue lymphoma. Am J Pathol, 2005. 167(3): p. 797-812.

72. Watanabe, T., et al., Helicobacter pylori infection induces gastric cancer in mongolian gerbils. Gastroenterology, 1998. 115(3): p. 642-8.
73. Rossi, G., et al., A conventional beagle dog model for acute and chronic infection with Helicobacter pylori. Infect Immun, 1999. 67(6): p. 3112-3120.
74. Bäckhed, F., et al., Gastric mucosal recognition of Helicobacter pylori is independent of Toll-like receptor 4. J Inf Dis, 2003. 187(5): p. 829-836.
75. Maeda, S., et al., Distinct mechanism of Helicobacter pylori-mediated NF-κB activation between gastric cancer cells and monocytic cells. J Biol Chem, 2001. 276(48): p. 44856-44864.
76. Bodger, K. and J.E. Crabtree, Helicobacter pylori and gastric inflammation. Br Med Bull, 1998. 54(1): p. 139-150.
77. Smith, M.F., et al., Toll-like receptor (TLR) 2 and TLR5, but not TLR4, are required for Helicobacter pylori-induced NF-κB activation and chemokine expression by epithelial cells. J Biol Chem, 2003. 278(35): p. 32552-32560.
78. Kawahara, T., et al., Type I Helicobacter pylori lipopolysaccharide stimulates Toll-like receptor 4 and activates mitogen oxidase 1 in gastric pit cells. Infect Immun, 2001. 69(7): p. 4382-4389.
79. Su, B., et al., Helicobacter pylori activates Toll-like receptor 4 expression in gastrointestinal epithelial cells. Infect Immun, 2003. 71(6): p. 3496-3502.
80. Lee, S.K., et al., Helicobacter pylori flagellins have very low intrinsic activity to stimulate human gastric epithelial cells via TLR5. Microb Infect, 2003. 5(15): p. 1345-1356.
81. Obonyo, M., et al., Deficiencies of myeloid differentiation factor 88, Toll-like receptor 2 (TLR2), or TLR4 produce specific defects in macrophage cytokine secretion induced by Helicobacter pylori. Infect Immun, 2007. 75(5): p. 2408-2414.
82. Mandell, L., et al., Intact Gram-negative Helicobacter pylori, Helicobacter felis, and Helicobacter hepaticus bacteria activate innate immunity via Toll-like receptor 2 but not Toll-like receptor 4. Infect Immun, 2004. 72(11): p. 6446-6454.
83. Gewirtz, A.T., et al., Helicobacter pylori flagellin evades Toll-like receptor 5-mediated innate immunity. J Inf Dis, 2004. 189(10): p. 1914-1920.
84. Rad, R., et al., Toll-like receptor-dependent activation of antigen-presenting cells affects adaptive immunity to Helicobacter pylori. Gastroenterology, 2007. 133(1): p. 150-163.e3.
85. Rad, R., et al., Extracellular and intracellular pattern recognition receptors cooperate in the recognition of Helicobacter pylori. Gastroenterology, 2009. 136(7): p. 2247-2257.
86. Viala, J., et al., Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. Nat Immunol, 2004. 5(11): p. 1166-1174.
87. Kaparakis, M., et al., Bacterial membrane vesicles deliver peptidoglycan to NOD1 in epithelial cells. Cell Microbiol, 2010. 12(3): p. 372-385.
88. Watanabe, T., et al., NOD1-mediated mucosal host defense against Helicobacter pylori. Int J Inflam, 2010. 2010: p. 476482.
89. Grubman, A., et al., The innate immune molecule, NOD1, regulates direct killing of Helicobacter pylori by antimicrobial peptides. Cell Microbiol, 2010. 12(5): p. 626-639.
90. Mariathasan, S. and D.M. Monack, Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation. Nat Rev Immunol, 2007. 7(1): p. 31-40.
91. Mariathasan, S., et al., Innate immunity against Francisella tularensis is dependent on the ASC/caspase-1 axis. J Exp Med, 2005. 202(8): p. 1043-1049.
92. Amer, A., et al., Regulation of Legionella phagosome maturation and infection through flagellin and host Ipaf. J Biol Chem, 2006. 281(46): p. 35217-35223.
93. Dunne, A., et al., Inflammasome activation by adenylate cyclase toxin directs Th17 responses and protection against Bordetella pertussis. J Immunol, 2010. 185(3): p. 1711-1719.
94. Suzuki, T., et al., Differential regulation of caspase-1 activation, pyroptosis, and autophagy via Ipaf and ASC in Shigella-infected macrophages. PLoS Pathog, 2007. 3(8): p. e111.
95. Brodsky, I.E. and D. Monack, NLR-mediated control of inflammasome assembly in the host response against bacterial pathogens. Sem Immunol, 2009. 21(4): p. 199-207.
96. Franchi, L., et al., The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. Nat Immunol, 2009. 10(3): p. 241-247.

97. Basak, C., et al., NF-kappaB- and C/EBPbeta-driven interleukin-1beta gene expression and PAK1-mediated caspase-1 activation play essential roles in interleukin-1beta release from Helicobacter pylori lipopolysaccharide-stimulated macrophages. J Biol Chem, 2005. 280(6): p. 4279-4288.
98. Tan, M.P., et al., CD8+ T cells are associated with severe gastritis in Helicobacter pylori-infected mice in the absence of CD4+ T cells. Infect Immun, 2008. 76(3): p. 1289-1297.
99. Bamford, K.B., et al., Lymphocytes in the human gastric mucosa during Helicobacter pylori have a T helper cell 1 phenotype. Gastroenterology, 1998. 114(3): p. 482-492.
100. Quiding-Järbrink, M., et al., CD4+ and CD8+ T cell responses in Helicobacter pylori-infected individuals. Clin Exp Immunol, 2001. 123(1): p. 81-87.
101. Lindholm, C., et al., Local cytokine response in Helicobacter pylori-infected subjects. Infect Immun, 1998. 66(12): p. 5964-71.
102. Karttunen, R.A., et al., Expression of mRNA for interferon-gamma, interleukin-10, and interleukin-12 (p40) in normal gastric mucosa and in mucosa infected with Helicobacter pylori. Scand J Gastroenterol, 1997. 32(1): p. 22-27.
103. Mohammadi, M., et al., Helicobacter-specific cell-mediated immune responses display a predominant Th1 phenotype and promote a delayed-type hypersensitivity response in the stomachs of mice. J Immunol, 1996. 156(12): p. 4729-38.
104. Smythies, L.E., et al., Helicobacter pylori-induced mucosal inflammation is Th1 mediated and exacerbated in IL-4, but not IFN-gamma, gene-deficient mice. J Immunol, 2000. 165(2): p. 1022-9.
105. Guiney, D.G., P. Hasegawa, and S.P. Cole, Helicobacter pylori preferentially induces interleukin 12 (IL-12) rather than IL-6 or IL-10 in human dendritic cells. Infect Immun, 2003. 71(7): p. 4163-4166.
106. Meyer, F., K.T. Wilson, and S.P. James, Modulation of innate cytokine responses by products of Helicobacter pylori. Infect Immun, 2000. 68(11): p. 6265-6272.
107. Mohammadi, M., et al., Murine CD4 T-cell response to Helicobacter infection: TH1 cells enhance gastritis and TH2 cells reduce bacterial load. Gastroenterology, 1997. 113(6): p. 1848-57.
108. Eaton, K., M. Mefford, and T. Thevenot, The role of T cell subsets and cytokines in the pathogenesis of Helicobacter pylori gastritis in mice. J Immunol, 2001. 166(12): p. 7456-61.
109. Ye, P., et al., Interleukin-17 and lung host defense against Klebsiella pneumoniae infection. Am J Respir Cell Mol Biol, 2001. 25(3): p. 335-340.
110. Matsuzaki, G. and M. Umemura, Interleukin-17 as an effector molecule of innate and acquired immunity against infections. Microbiol Immunol, 2007. 51(12): p. 1139-47.
111. Nakae, S., et al., IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist. Proc Natl Acad Sci U S A, 2003. 100(10): p. 5986-5990.
112. Langrish, C.L., et al., IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med, 2005. 201(2): p. 233-240.
113. Park, H., et al., A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol, 2005. 6(11): p. 1133-1141.
114. Lundgren, A., et al., Mucosal FOXP3-expressing CD4+ CD25high regulatory T cells in Helicobacter pylori-infected patients. Infect Immun, 2005. 73(1): p. 523-531.
115. Rad, R., et al., CD25+/Foxp3+ T cells regulate gastric inflammation and Helicobacter pylori colonization in vivo. Gastroenterology, 2006. 131(2): p. 525-537.
116. Raghavan, S., et al., Absence of CD4+CD25+ regulatory T cells is associated with a loss of regulation leading to increased pathology in Helicobacter pylori-infected mice. Clin Exp Immunol, 2003. 132(3): p. 393-400.
117. Akhiani, A.A., et al., Helicobacter pylori-specific antibodies impair the development of gastritis, facilitate bacterial colonization, and counteract resistance against infection. J Immunol, 2004. 172(8): p. 5024-5033.
118. Bussiere, F.I., et al., Low multiplicity of infection of Helicobacter pylori suppresses apoptosis of B lymphocytes. Cancer Res, 2006. 66(13): p. 6834-6842.
119. Yamanishi, S., et al., Implications for induction of autoimmunity via activation of B-1 cells by Helicobacter pylori urease. Infect Immun, 2006. 74(1): p. 248-256.

120. Wilson, K.T. and J.E. Crabtree, Immunology of Helicobacter pylori: insights into the failure of the immune response and perspectives on vaccine studies. Gastroenterology, 2007. 133(1): p. 288-308.
121. Ferrero, R.L., et al., The GroES homolog of Helicobacter pylori confers protective immunity against mucosal infection in mice. Proc Natl Acad Sci U S A, 1995. 92(14): p. 6499-6503.
122. Marchetti, M., et al., Protection against Helicobacter pylori infection in mice by intragastric vaccination with H. pylori antigens is achieved using a non-toxic mutant of E. coli heat-labile enterotoxin (LT) as adjuvant. Vaccine, 1998. 16(1): p. 33-37.
123. Ermak, T.H., et al., Immunization of mice with urease vaccine affords protection against Helicobacter pylori infection in the absence of antibodies and is mediated by MHC class II-restricted responses. J Exp Med, 1998. 188(12): p. 2277-2288.
124. Ermak, T.H., et al., Gastritis in urease-immunized mice after Helicobacter felis challenge may be due to residual bacteria. Gastroenterology, 1997. 113(4): p. 1118-1128.
125. Ghiara, P., et al., Therapeutic intragastric vaccination against Helicobacter pylori in mice eradicates an otherwise chronic infection and confers protection against reinfection. Infect Immun, 1997. 65(12): p. 4996-5002.
126. Pappo, J., et al., Effect of oral immunization with recombinant urease on murine Helicobacter felis gastritis. Infect Immun, 1995. 63(4): p. 1246-1252.
127. Raghavan, S., A.M. Svennerholm, and J. Holmgren, Effects of oral vaccination and immunomodulation by Cholera toxin on experimental Helicobacter pylori infection, reinfection, and gastritis. Infect Immun, 2002. 70(8): p. 4621-4627.
128. Radcliff, F., et al., Catalase, a novel antigen for Helicobacter pylori vaccination. Infect Immun, 1997. 65(11): p. 4668-4674.
129. Michetti, P., et al., Immunization of BALB/c mice against Helicobacter felis infection with Helicobacter pylori urease. Gastroenterology, 1994. 107(4): p. 1002-11.
130. Shi, T., et al., Intranasal CpG-oligodeoxynucleotide is a potent adjuvant of vaccine against Helicobacter pylori, and T helper 1 type response and interferon-γ correlate with the protection. Helicobacter, 2005. 10(1): p. 71-79.
131. Guy, B., et al., Systemic immunization with urease protects mice against Helicobacter pylori infection. Vaccine, 1998. 16(8): p. 850-856.
132. Gottwein, J.M., et al., Protective anti-Helicobacter immunity is induced with aluminum hydroxide or Complete Freund's Adjuvant by systemic immunization. J Inf Dis, 2001. 184(3): p. 308-314.
133. Rossi, G., et al., Therapeutic vaccination against Helicobacter pylori in the beagle dog experimental model: Safety, immunogenicity, and efficacy. Infect Immun, 2004. 72(6): p. 3252-3259.
134. Eisenberg, J.C., et al., Protective efficacy of anti-Helicobacter pylori immunity following systemic immunization of neonatal mice. Infect Immun, 2003. 71(4): p. 1820-1827.
135. Sommer, F., et al., Systemic Th1 immunization of mice against Helicobacter pylori infection with CpG oligodeoxynucleotides as adjuvants does not protect from infection but enhances gastritis. Infect Immun, 2004. 72(2): p. 1029-1035.
136. Angelakopoulos, H. and E.L. Hohmann, Pilot study of phoP/phoQ-deleted Salmonella enterica serovar Typhimurium expressing Helicobacter pylori urease in adult volunteers. Infect Immun, 2000. 68(4): p. 2135-2141.
137. Bumann, D., et al., Safety and immunogenicity of live recombinant Salmonella enterica serovar Typhi Ty21a expressing urease A and B from Helicobacter pylori in human volunteers. Vaccine, 2001. 20(5-6): p. 845-852.
138. DiPetrillo, M.D., et al., Safety and immunogenicity of phoP/phoQ-deleted Salmonella typhi expressing Helicobacter pylori urease in adult volunteers. Vaccine, 1999. 18(5-6): p. 449-459.
139. Nyström, J. and A.-M. Svennerholm, Oral immunization with HpaA affords therapeutic protective immunity against H. pylori that is reflected by specific mucosal immune responses. Vaccine, 2007. 25(14): p. 2591-2598.

140. Wu, C., et al., Protection against Helicobacter pylori infection in Mongolian gerbil by intragastric or intramuscular administration of H. pylori multicomponent vaccine. Helicobacter, 2008. 13(3): p. 191-199.
141. Skene, C.D., C. Doidge, and P. Sutton, Evaluation of ISCOMATRIX and ISCOM vaccines for immunisation against Helicobacter pylori. Vaccine, 2008. 26(31): p. 3880-3884.
142. Weltzin, R., et al., Parenteral adjuvant activities of Escherichia coli heat-labile toxin and its B subunit for immunization of mice against gastric Helicobacter pylori infection. Infect Immun, 2000. 68(5): p. 2775-2782.
143. Zhao, W., W. Wu, and X. Xu, Oral vaccination with liposome-encapsulated recombinant fusion peptide of urease B epitope and cholera toxin B subunit affords prophylactic and therapeutic effects against H. pylori infection in BALB/c mice. Vaccine, 2007. 25(44): p. 7664-7673.
144. Petrovsky, N. and J.C. Aguilar, Vaccine adjuvants: Current state and future trends. Immunol Cell Biol, 2004. 82(5): p. 488-496.
145. Raghavan, S., et al., Orally Administered CpG Oligodeoxynucleotide Induces Production of CXC and CC Chemokines in the Gastric Mucosa and Suppresses Bacterial Colonization in a Mouse Model of Helicobacter pylori Infection. Infect. Immun., 2003. 71(12): p. 7014-7022.
146. Raghavan, S., et al., Orally administered CpG oligodeoxynucleotide induces production of CXC and CC chemokines in the gastric mucosa and suppresses bacterial colonization in a mouse model of Helicobacter pylori infection. Infect Immun, 2003. 71(12): p. 7014-7022.
147. Zhang, M., et al., Helicobacter pylori-pulsed dendritic cells induce H. pylori-specific immunity in mice. Helicobacter, 2008. 13(3): p. 200-208.
148. Hickey, D.K., et al., Transcutaneous immunization with novel lipid-based adjuvants induces protection against gastric Helicobacter pylori infection. Vaccine, 2009. 27(50): p. 6983-6990.
149. Czinn, S.J. and T. Blanchard, Vaccinating against Helicobacter pylori infection. Nat Rev Gastroenterol Hepatol, 2011. 8(3): p. 133-40.
150. Kotloff, K.L., et al., Safety and immunogenicity of oral inactivated whole-cell Helicobacter pylori vaccine with adjuvant among volunteers with or without subclinical infection. Infect Immun, 2001. 69(6): p. 3581-3590.
151. Michetti, P., et al., Oral immunization with urease and Escherichia coli heat-labile enterotoxin is safe and immunogenic in Helicobacter pyloriâ€“infected adults. Gastroenterology, 1999. 116(4): p. 804-812.
152. Banerjee, S., et al., Safety and efficacy of low dose Escherichia coli enterotoxin adjuvant for urease based oral immunisation against Helicobacter pylori in healthy volunteers. Gut, 2002. 51(5): p. 634-640.
153. Sougioultzis, S., et al., Safety and efficacy of E. coli enterotoxin adjuvant for urease-based rectal immunization against Helicobacter pylori. Vaccine, 2002. 21(3-4): p. 194-201.
154. Aebischer, T., et al., Correlation of T cell response and bacterial clearance in human volunteers challenged with Helicobacter pylori revealed by randomised controlled vaccination with Ty21a-based Salmonella vaccines. Gut, 2008. 57(8): p. 1065-1072.
155. Malfertheiner, P., et al., Safety and immunogenicity of an intramuscular Helicobacter pylori vaccine in noninfected volunteers: A phase I study. Gastroenterology, 2008. 135(3): p. 787-795.
156. Ferrero, R.L., J.M. Thiberge, and A. Labigne, Local immunoglobulin G antibodies in the stomach may contribute to immunity against Helicobacter infection in mice. Gastroenterology, 1997. 113(1): p. 185-194.
157. Czinn, S., A. Cai, and J. Nedrud, Protection of germ-free mice from infection by Helicobacter felis after active oral or passive IgA immunization. Vaccine, 1993. 11(6): p. 637-42.
158. Marnila, P., et al., Prevention and suppression of Helicobacter felis infection in mice using colostral preparation with specific antibodies. Helicobacter, 2003. 8(3): p. 192-201.
159. Nomura, S., et al., Effect of dietary anti-urease immunoglobulin Y on Helicobacter pylori infection in Mongolian gerbils. Helicobacter, 2005. 10(1): p. 43-52.
160. Gorrell, R.J. and R.M. Robins-Browne, Antibody-mediated protection against infection with Helicobacter pylori in a suckling mouse model of passive immunity. Infect Immun, 2009. 77(11): p. 5116-5129.

161. Thomas, J.E., et al., Specific immunoglobulin A antibodies in maternal milk and delayed Helicobacter pylori colonization in Gambian infants. Clin Infect Dis, 2004. 39(8): p. 1155-1160.
162. Garhart, C.A., et al., Vaccine-induced protection against Helicobacter pylori in mice lacking both antibodies and interleukin-4. Infect Immun, 2003. 71(6): p. 3628-3633.
163. Sutton, P., et al., Therapeutic immunization against Helicobacter pylori infection in the absence of antibodies. Immunol Cell Biol, 2000. 78(1): p. 28-30.
164. Akhiani, A.A., et al., IgA antibodies impair resistance against Helicobacter pylori infection: studies on immune evasion in IL-10-deficient mice. J Immunol, 2005. 174(12): p. 8144-8153.
165. Pappo, J., et al., Helicobacter pylori infection in immunized mice lacking major histocompatibility complex class I and class II functions. Infect Immun, 1999. 67(1): p. 337-341.
166. Marinaro, M., et al., Mucosal adjuvant effect of cholera toxin in mice results from induction of T helper 2 (Th2) cells and IL-4. J Immunol, 1995. 155(10): p. 4621-4629.
167. Yamamoto, S., et al., A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. Proc Natl Acad Sci U S A, 1997. 94(10): p. 5267-72.
168. Lavelle, E.C., et al., Effects of cholera toxin on innate and adaptive immunity and its application as an immunomodulatory agent. J Leukoc Biol, 2004. 75(5): p. 756-763.
169. Xu-Amano, J., et al., Helper T cell subsets for immunoglobulin A responses: oral immunization with tetanus toxoid and cholera toxin as adjuvant selectively induces Th2 cells in mucosa associated tissues. J Exp Med, 1993. 178(4): p. 1309-20.
170. Braun, M.C., et al., Cholera toxin suppresses interleukin (IL)-12 production and IL-12 receptor β1 and β2 chain expression. J Exp Med, 1999. 189(3): p. 541-552.
171. Akhiani, A.A., et al., Protection against Helicobacter pylori infection following immunization is IL-12-dependent and mediated by Th1 cells. J Immunol, 2002. 169(12): p. 6977-6984.
172. Garhart, C.A., et al., Vaccine-induced reduction of Helicobacter pylori colonization in mice is interleukin-12 dependent but gamma interferon and inducible nitric oxide synthase independent. Infect Immun, 2003. 71(2): p. 910-921.
173. Taylor, J.M., et al., Possible correlates of long-term protection against Helicobacter pylori following systemic or combinations of mucosal and systemic immunizations. Infect Immun, 2007. 75(7): p. 3462-3469.
174. Taylor, J.M., et al., Effects of a Th1- versus a Th2-biased immune response in protection against Helicobacter pylori challenge in mice. Microb Pathog, 2008. 44(1): p. 20-27.
175. Panthel, K., G. Faller, and R. Haas, Colonization of C57BL/6J and BALB/c wild-type and knockout mice with Helicobacter pylori: effect of vaccination and implications for innate and acquired immunity. Infect Immun, 2003. 71(2): p. 794-800.
176. Kaparakis, M., et al., CD4+ CD25+ regulatory T cells modulate the T-cell and antibody responses in Helicobacter-infected BALB/c mice. Infect Immun, 2006. 74(6): p. 3519-3529.
177. Kaparakis, M., et al., Macrophages are mediators of gastritis in acute Helicobacter pylori infection in C57BL/6 mice. Infect Immun, 2008. 76(5): p. 2235-2239.
178. Caruso, R., et al., IL-23-mediated regulation of IL-17 production in Helicobacter pylori-infected gastric mucosa. Eur J Immunol, 2008. 38(2): p. 470-478.
179. Shiomi, S., et al., IL-17 is involved in Helicobacter pylori-induced gastric inflammatory responses in a mouse model. Helicobacter, 2008. 13(6): p. 518-524.
180. DeLyria, E.S., R.W. Redline, and T.G. Blanchard, Vaccination of mice against H pylori induces a strong Th-17 response and immunity that is neutrophil dependent. Gastroenterology, 2009. 136(1): p. 247-256.
181. Velin, D., et al., Interleukin-17 is a critical mediator of vaccine-induced reduction of Helicobacter infection in the mouse model. Gastroenterology, 2009. 136(7): p. 2237-2246.e1.
182. Flach, C.-F., et al., Proinflammatory cytokine gene expression in the stomach correlates with vaccine-induced protection against Helicobacter pylori infection in mice: an important role for interleukin-17 during the effector phase. Infect Immun, 2011. 79(2): p. 879-886.
183. Datta, S.K., et al., Mucosal adjuvant activity of cholera toxin requires Th17 cells and protects against inhalation anthrax. Proc Natl Acad Sci U S A, 2010. 107(23): p. 10638-10643.

184. Okamura, H., et al., Cloning of a new cytokine that induces IFN-gamma production by T cells. Nature, 1995. 378(6552): p. 88-91.
185. Akhiani, A.A., K. Schon, and N. Lycke, Vaccine-induced immunity against Helicobacter pylori infection is impaired in IL-18-deficient mice. J Immunol, 2004. 173(5): p. 3348-3356.
186. Ismail, H.F., et al., Depletion of neutrophils in IL-$10^{-/-}$ mice delays clearance of gastric Helicobacter infection and decreases the Th1 immune response to Helicobacter. J Immunol, 2003. 170(7): p. 3782-3789.
187. Velin, D., et al., Mast cells are critical mediators of vaccine-induced Helicobacter clearance in the mouse model. Gastroenterology, 2005. 129(1): p. 142-155.
188. Ding, H., et al., Partial protection against Helicobacter pylori in the absence of mast cells in mice. Infect Immun, 2009. 77(12): p. 5543-5550.
189. Kao, J.Y., et al., Helicobacter pylori immune escape is mediated by dendritic cell-induced Treg skewing and Th17 suppression in mice. Gastroenterology, 2010. 138(3): p. 1046-1054.
190. Drakes, M.L., S.J. Czinn, and T.G. Blanchard, Regulation of murine dendritic cell immune responses by Helicobacter felis antigen. Infect Immun, 2006. 74(8): p. 4624-4633.
191. Sayi, A., et al., TLR-2–activated B cells suppress Helicobacter-induced preneoplastic gastric immunopathology by inducing T regulatory-1 cells. J Immunol, 2011. 186(2): p. 878-890.
192. Cella, M., et al., Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. J Exp Med, 1996. 184(2): p. 747-752.
193. De Gregorio, E., E. Tritto, and R. Rappuoli, Alum adjuvanticity: Unraveling a century old mystery. Eur J Immunol, 2008. 38(8): p. 2068-2071.
194. Sokolovska, A., S.L. Hem, and H. HogenEsch, Activation of dendritic cells and induction of CD4+ T cell differentiation by aluminum-containing adjuvants. Vaccine, 2007. 25(23): p. 4575-4585.
195. Lindblad, E.B., Aluminium adjuvants--in retrospect and prospect. Vaccine, 2004. 22(27-28): p. 3658-3668.
196. Nyström-Asklin, J., J. Adamsson, and A.M. Harandi, The adjuvant effect of CpG oligodeoxynucleotide linked to the non-toxic B subunit of Cholera toxin for induction of immunity against H. pylori in mice. Scand J Immunol, 2008. 67(5): p. 431-440.
197. Martin-Fontecha, A., et al., Induced recruitment of NK cells to lymph nodes provides IFN-[gamma] for TH1 priming. Nat Immunol, 2004. 5(12): p. 1260-1265.
198. Hemmi, H., et al., Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat Immunol, 2002. 3(2): p. 196-200.
199. Jurk, M., et al., Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. Nat Immunol, 2002. 3(6): p. 499-499.
200. McLachlan, J.B., et al., Mast cell activators: a new class of highly effective vaccine adjuvants. Nat Med, 2008. 14(5): p. 536-541.
201. McGowen, A.L., et al., The mast cell activator compound 48/80 is safe and effective when used as an adjuvant for intradermal immunization with Bacillus anthracis protective antigen. Vaccine, 2009. 27(27): p. 3544-3552.
202. Trumpfheller, C., et al., The microbial mimic poly IC induces durable and protective CD4+ T cell immunity together with a dendritic cell targeted vaccine. Proc Natl Acad Sci U S A, 2008. 105(7): p. 2574-2579.
203. Sullivan, B.A. and M. Kronenberg, Activation or anergy: NKT cells are stunned by alpha-galactosylceramide. J Clin Invest, 2005. 115(9): p. 2328-2329.
204. Ko, S.-Y., et al., Alpha-galactosylceramide can act as a nasal vaccine adjuvant inducing protective immune responses against viral infection and tumor. J Immunol, 2005. 175(5): p. 3309-3317.
205. Choi, Y.S., et al., Alpha-Galactosylceramide enhances the protective and therapeutic effects of tumor cell based vaccines for ovarian tumors. Vaccine, 2008. 26(46): p. 5855-5863.
206. Gonzalez-Aseguinolaza, G., et al., Natural killer T cell ligand α-galactosylceramide enhances protective immunity induced by malaria vaccines. J Exp Med, 2002. 195(5): p. 617-624.

207. Dondji, B., et al., Intradermal NKT cell activation during DNA priming in heterologous prime-boost vaccination enhances T cell responses and protection against Leishmania. Eur J Immunol, 2008. 38(3): p. 706-719.
208. Agger, E.M., et al., Cationic liposomes formulated with synthetic mycobacterial cordfactor (CAF01): A versatile adjuvant for vaccines with different immunological requirements. PLoS ONE, 2008. 3(9): p. e3116.
209. Werninghaus, K., et al., Adjuvanticity of a synthetic cord factor analogue for subunit Mycobacterium tuberculosis vaccination requires FcRgamma-Syk-Card9-dependent innate immune activation. J Exp Med, 2009. 206(1): p. 89-97.
210. Korsholm, K.S., et al., The adjuvant mechanism of cationic dimethyldioctadecylammonium liposomes. Immunology, 2007. 121(2): p. 216-226.
211. Kamath, A.T., et al., A liposome-based mycobacterial vaccine induces potent adult and neonatal multifunctional T cells through the exquisite targeting of dendritic cells. PLoS ONE, 2009. 4(6): p. e5771.
212. Korsholm, K.S., et al., T-helper 1 and T-helper 2 adjuvants induce distinct differences in the magnitude, quality and kinetics of the early inflammatory response at the site of injection. Immunology, 2010. 129(1): p. 75-86.
213. Schoenen, H., et al., Cutting Edge: Mincle is essential for recognition and adjuvanticity of the mycobacterial cord factor and its synthetic analog trehalose-dibehenate. J Immunol, 2010. 184(6): p. 2756-2760.
214. Kool, M., et al., Cutting Edge: Alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome. J Immunol, 2008. 181(6): p. 3755-3759.
215. Eisenbarth, S.C., et al., Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. Nature, 2008. 453(7198): p. 1122-1126.
216. LeibundGut-Landmann, S., et al., Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. Nat Immunol, 2007. 8(6): p. 630-638.
217. Sawai, N., et al., Role of gamma interferon in Helicobacter pylori-induced gastric inflammatory responses in a mouse model. Infect Immun, 1999. 67(1): p. 279-85.
218. Toller, I.M., et al., Prostaglandin E2 prevents Helicobacter-induced gastric preneoplasia and facilitates persistent infection in a mouse model. Gastroenterology, 2010. 138(4): p. 1455-1467.e4.
219. Toller, I.M., et al., Inhibition of ADP ribosylation prevents and cures Helicobacter-induced gastric preneoplasia. Cancer Res, 2010. 70(14): p. 5912-5922.
220. Kamradt, A.E., et al., Helicobacter pylori infection in wild-type and cytokine-deficient C57BL/6 and BALB/c mouse mutants. Microb Infect, 2000. 2(6): p. 593-7.
221. Sutton, P., et al., Post-immunisation gastritis and Helicobacter infection in the mouse: a long term study. Gut, 2001. 49(4): p. 467-473.
222. Raghavan, S., et al., Protection against experimental Helicobacter pylori infection after immunization with inactivated H. pylori whole-cell vaccines. Infect Immun, 2002. 70(11): p. 6383-6388.
223. Garhart, C.A., et al., Clearance of Helicobacter pylori infection and resolution of postimmunization gastritis in a kinetic study of prophylactically immunized mice. Infect Immun, 2002. 70(7): p. 3529-3538.
224. Eaton, K.A. and M.E. Mefford, Cure of Helicobacter pylori infection and resolution of gastritis by adoptive transfer of splenocytes in mice. Infect Immun, 2001. 69(2): p. 1025-1031.
225. Jeremy, A.H.T., et al., Protection against Helicobacter pylori infection in the Mongolian gerbil after prophylactic vaccination. Microb Infect, 2006. 8(2): p. 340-346.
226. van de Veerdonk, F.L., et al., Inflammasome activation and IL-1beta and IL-18 processing during infection. Trends Immunol, 2011. 32(3): p. 110-116.
227. Chung, Y., et al., Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. Immunity, 2009. 30(4): p. 576-587.
228. Dinarello, C., IL-18: A TH1-inducing, proinflammatory cytokine and new member of the IL-1 family. J Allergy Clin Immunol, 1999. 103((1 Pt 1)): p. 11-24.

229. Dupaul-Chicoine, J., et al., Control of intestinal homeostasis, colitis, and colitis-associated colorectal cancer by the inflammatory caspases. Immunity, 2010. 32(3): p. 367-378.
230. Zaki, M.H., et al., The NLRP3 inflammasome protects against loss of epithelial integrity and mortality during experimental colitis. Immunity, 2010. 32(3): p. 379-391.
231. Zaki, M.H., et al., IL-18 production downstream of the Nlrp3 inflammasome confers protection against colorectal tumor formation. J Immunol, 2010. 185(8): p. 4912-4920.
232. Kanai, T., et al., Macrophage-derived IL-18-mediated intestinal inflammation in the murine model of Crohn's disease. Gastroenterology, 2001. 121(4): p. 875-888.
233. Siegmund, B., et al., IL-1beta-converting enzyme (caspase-1) in intestinal inflammation. Proc Natl Acad Sci U S A, 2001. 98(23): p. 13249-13254.
234. Maldonado, R. and U. von Andrian, How tolerogenic dendritic cells induce regulatory T cells. Adv Immunol, 2010. 108: p. 111-65.
235. Fainaru, O., et al., TGFbeta-dependent gene expression profile during maturation of dendritic cells. Genes Immun, 2007. 8(3): p. 239-244.
236. Veldhoen, M., et al., TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity, 2006. 24(2): p. 179-189.
237. Yang, X.O., et al., Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. Immunity, 2008. 29(1): p. 44-56.
238. Xu, L., et al., Cutting Edge: Regulatory T cells induce CD4+CD25-Foxp3- T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-β. J Immunol, 2007. 178(11): p. 6725-6729.
239. Chen, Y., et al., Foxp3+ regulatory T cells promote T helper 17 cell development in vivo through regulation of interleukin-2. Immunity, 2011. 34(3): p. 409-421.
240. Deknuydt, F., et al., IL-1beta and IL-2 convert human Treg into TH17 cells. Clin Immunol, 2009. 131(2): p. 298-307.
241. Li, L., J. Kim, and V.A. Boussiotis, IL-1β–mediated signals preferentially drive conversion of regulatory T cells but not conventional T cells into IL-17–producing cells. J Immunol, 2010. 185(7): p. 4148-4153.
242. Harrington, L.E., et al., Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol, 2005. 6(11): p. 1123-1132.
243. Pejnovic, N., et al., Increased atherosclerotic lesions and Th17 in interleukin-18 deficient apolipoprotein E-knockout mice fed high-fat diet. Mol Immunol, 2009. 47(1): p. 37-45.
244. Rupnow, M.F.T., et al., Cost-effectiveness of a potential prophylactic Helicobacter pylori vaccine in the United States. J Inf Dis, 2009. 200(8): p. 1311-1317.
245. Yeh, J., et al., Effects of Helicobacter pylori infection and smoking on gastric cancer incidence in China: a population-level analysis of trends and projections. Canc Causes Contr, 2009. 20(10): p. 2021-2029.
246. Raghavan, S., et al., Sublingual immunization protects against Helicobacter pylori infection and induces T and B cell responses in the stomach. Infect Immun, 2010. 78(10): p. 4251-4260.
247. Vujanic, A., et al., Combined mucosal and systemic immunity following pulmonary delivery of ISCOMATRIX adjuvanted recombinant antigens. Vaccine, 2010. 28(14): p. 2593-2597.
248. Blaser, M.J., Helicobacter pylori and esophageal disease: Wake-up call? Gastroenterology, 2010. 139(6): p. 1819-1822.
249. Reibman, J., et al., Asthma Is inversely associated with Helicobacter pylori status in an urban population. PLoS ONE, 2008. 3(12): p. e4060.
250. Chen, Y. and M.J. Blaser, Inverse associations of Helicobacter pylori with asthma and allergy. Arch Intern Med, 2007. 167(8): p. 821-827.
251. Tatsuguchi, A., et al., Effect of Helicobacter pylori infection on ghrelin expression in human gastric mucosa. Am J Gastroenterol, 2004. 99(11): p. 2121-2127.
252. Weigt, J. and P. Malfertheiner, Influence of Helicobacter pylori on gastric regulation of food intake. Curr Opin Clin Nutr Metab Care, 2009. 12(5): p. 522-525.
253. Huang, X., et al., Iron deficiency anaemia can be improved after eradication of Helicobacter pylori. Postgrad Med J, 2010. 86(1015): p. 272-278.

254. Yuan, W., et al., Iron deficiency anemia in Helicobacter pylori infection: meta-analysis of randomized controlled trials. Scand J Gastroenterol, 2010. 45(6): p. 665-676.
255. Gasbarrini, A., et al., Regression of autoimmune thrombocytopenia after eradication of Helicobacter pylori. Lancet, 1998. 352(9131): p. 878-878.
256. Kodama, M., et al., Immune response to CagA protein is associated with improved platelet count after Helicobacter pylori eradication in patients with idiopathic thrombocytopenic purpura. Helicobacter, 2007. 12(1): p. 36-42.
257. Ferrara, M., L. Capozzi, and R. Russo, Effect of Helicobacter pylori eradication on platelet count in children with chronic idiopathic thrombocytopenic purpura. Hematology, 2009. 14(5): p. 282-285.

7. ACKNOWLEDGEMENTS

This dissertation would not have succeeded without the contribution of many others.

First and foremost I wish to thank Anne for the invaluable contribution throughout. By consigning these projects to me, guiding and supporting me with always new ideas and incentives you are an important driving force behind this work. I admire how you have made the lab grow scientifically and in size in such a short time and I am sure it will continue like that.

I am very grateful to the members of my thesis committee Burkhard Becher, Maries van den Broek and Roberto Speck for following my progress, critical discussions and constructive suggestions, during our meetings and beyond that.

Best thanks to our collaborators on the adjuvant project, Else-Marie Agger and Johanna Nyström-Asklin from the Statens Serum Institute in Denmark, for helpful comments and the prompt and ceaseless reagent supply. Thanks also to Gregor Fischer, Lubor Borsig, Daniel Pochetti and the rest of the crew for running and maintaining the animal facility, taking care of our mice and helping with any problems.

I am grateful to all IMCR members for the genial working atmosphere in the institute, and especially to the administrative and technical staff that enables us to concentrate entirely on research. Furthermore to Joe Jiricny, Cornelia Schauz and Bettina Rausch for providing support and a great scientific environment through the Cancer Biology PhD and URPP programs.

The Müller lab has big merits in making my doctorate possible and an unforgettable time. A huge thank you to all of its former and current members – Esther, Ayca, the Isas, Vanessa, Mats, Dani, Mara – for the good team spirit, fun times in and outside the lab, scientific input, collaborations and, last but not least, endless help with endless mouse studies… I will miss you, and the view on the cows.

Finally, I would like to express my gratitude towards my family, friends and Joris for the continuous encouragement from near and far and diverting my attention also to other things.

8. CURRICULUM VITAE

PERSONAL DETAILS

Name:	Iris Marion Hitzler
Date of birth:	July 19, 1983
Nationality:	German

EDUCATION

since 11/2007	**University of Zurich, Institute of Molecular Cancer Research** employed as PhD student in the group of Prof. Anne Müller PhD thesis: *Immune mechanisms governing infection control and pathogenesis of Helicobacter pylori and prospects for a human vaccine*
01/2007-08/2007	**La Jolla Institute of Allergy and Immunology, San Diego, USA** Master's thesis in the group of Prof. Matthias von Herrath: *Characterization of antigen-specific regulatory T cells and their use for immunotherapy of type 1 diabetes*
07/2006-08/2006	**AMGEN Research GmbH, Regensburg, Germany** Internship in the field of high-throughput screening of small compounds
07/2005-08/2005	**Queen's University, School of Pharmacy, Belfast, UK** Internship in the group of Prof. Koen Vandenbroeck on SNP genotyping
10/2004-09/2007	**École Supérieure de Biotechnologie, Strasbourg, France – joint program of the universities Strasbourg, Basel, Freiburg & Karlsruhe** MSc level degree in Biotechnology (Diplom)
10/2002-09/2004	**University of Regensburg, Germany** Pre-degree in Biology (Vordiplom)
09/1993-07/2002	**Albrecht-Altdorfer-Gymnasium, Regensburg, Germany** High school diploma (Abitur)

PUBLICATIONS

• Sayi A*, Kohler E*, Hitzler I*, Arnold I, Schwendener R, Rehrauer H, & Müller A. The $CD4^+$ T cell-mediated IFN-γ response to Helicobacter infection is essential for clearance and determines gastric cancer risk. *J Immunol.* 2009 (*equal contribution)

• Mueller A, Sayi A, Hitzler I. Protective and pathogenic functions of T-cells are inseparable during the Helicobacter-host interaction. *Discov Med*. 2009

• Toller IM, Hitzler I, Sayi A & Mueller A. Prostaglandin E2 prevents *Helicobacter*-induced gastric preneoplasia and facilitates persistent infection in a mouse model. *Gastroenterology.* 2010

• Hitzler I, Oertli M, Becher B, Agger E & Müller A. Dendritic cells prevent rather than promote immunity conferred by a *Helicobacter* vaccine using a mycobacterial adjuvant. *Gastroenterology.* 2011

• Arnold I, Hitzler I, Engler D, Oertli M, Agger E & Müller A. The C-terminally encoded, MHC class II-restricted T-cell antigenicity of the *Helicobacter pylori* virulence factor CagA promotes gastric preneoplasia. *J Immunol.* 2011

• Hitzler I, Sayi A, Kohler E, Hardt W & Müller A. Caspase-1 has pro-inflammatory and regulatory properties that are differentially mediated by processing of its substrates IL-1β and IL-18. *manuscript in preparation*

9. APPENDIX

Prostaglandin E2 prevents *Helicobacter*-induced gastric preneoplasia and facilitates persistent infection in a mouse model

article published in Gastroenterology, 2010

authors: Isabella Toller, Iris Hitzler, Ayca Sayi and Anne Müller

contributions: I contributed the figures 6 A-D, the real-time PCR data and helped with the revision. IT and AM planned, performed and analyzed experiments and prepared the manuscript.

Prostaglandin E2 Prevents *Helicobacter*-Induced Gastric Preneoplasia and Facilitates Persistent Infection in a Mouse Model

ISABELLA M. TOLLER, IRIS HITZLER, AYCA SAYI, and ANNE MUELLER

Institute of Molecular Cancer Research, University of Zürich, Zürich, Switzerland

BACKGROUND & AIMS: Persistent infection with the human pathogen *Helicobacter pylori* increases the risk of gastric cancer. In this study, we investigated the role of cyclooxygenase-2 (COX-2) and its main product, prostaglandin E_2 (PGE_2), in the development of *Helicobacter*-induced gastritis and gastric cancer precursor lesions. **METHODS:** We utilized mouse models of *Helicobacter*-induced gastric preneoplasia and vaccine-induced protection to study the effects of COX-2 inhibition and PGE_2 treatment on the induction of *Helicobacter*-specific immune responses and gastric premalignant immunopathology. **RESULTS:** COX-2 and PGE_2 are up-regulated upon *Helicobacter* infection in cultured epithelial cells and in the gastric mucosa of infected mice. Inhibition of COX-2 activity with celecoxib significantly accelerated early preneoplasia; conversely, systemic administration of synthetic PGE_2 prevented development of premalignant pathology and completely reversed preexisting lesions by suppressing interferon-γ production in the infected stomachs. The protective effect of PGE_2 was accompanied by increased *Helicobacter* colonization in all models. All in vivo effects were attributed to immunosuppressive effects of PGE_2 on $CD4^+$ T-helper 1 cells, which fail to migrate, proliferate, and secrete cytokines when exposed to PGE_2 in vitro and in vivo. T-cell inhibition was found to be due to silencing of interleukin-2 gene transcription, and could be overcome by supplementation with recombinant interleukin-2 in vitro and in vivo. **CONCLUSIONS: COX-2–dependent production of PGE_2 has an important immunomodulatory role during *Helicobacter* infection, preventing excessive local immune responses and the associated immunopathology by inhibiting the effector functions of pathogenic T-helper 1 cells.**

Keywords: Helicobacter pylori; Gastric Cancer; Cyclooxygenase; Prostaglandin E_2.

Persistent gastric colonization with the human pathogen *Helicobacter pylori* causes chronic active gastritis in infected individuals and has been linked epidemiologically to gastric and duodenal ulcers, gastric mucosa-associated lymphoid tissue lymphoma, and gastric cancer.[1–3] The chronic gastric inflammation induced by *H pylori* infection constitutes a prerequisite for the formation of preneoplastic and malignant lesions, both in humans and in rodent models of *Helicobacter*-induced disease.[4,5] The transformation from normal mucosa to gastric cancer occurs via a sequence of precursor lesions starting with atrophic gastritis, intestinal metaplasia, and dysplasia.[6] Using mouse models of experimental *Helicobacter* infection and vaccine-induced protection, we have shown recently that stomach-infiltrating $CD4^+CD25^-$ interferon-γ–secreting T cells play a dual role in the *Helicobacter*–host interaction. On the one hand, these cells are crucial in controlling the infection; on the other hand, they promote development of *Helicobacter*-induced gastric cancer precursor lesions, possibly through a direct effect of IFN-γ on gastric epithelial cells.[7]

The 2 processes of inflammation and carcinogenesis share a common molecular mediator, the cyclooxygenase (COX) enzymes. The COX-1 isoform is constitutively expressed in most cell types, whereas COX-2 is induced in response to proinflammatory stimuli, such as lipopolysaccharide, interleukin-1, and tumor necrosis factor–α.[8] The most important lipid product of COX-2, prostaglandin E_2 (PGE_2), exerts its effects by binding to the E-prostanoid receptors 1–4, which are expressed ubiquitously in most murine tissues.[9]

Several lines of evidence indicate that COX-2–dependent pathways play a role in carcinogenesis, especially in the gastrointestinal tract. Many solid tumors overexpress COX-2/PGE_2, and polymorphisms in the *COX-2* regulatory region determine the carrier's risk of developing several epithelial cancers.[10,11] The long-term low-level intake of nonsteroidal anti-inflammatory drugs inhibiting COX activity reduces the risk for colorectal cancer,[12,13] as well as gastric cancer.[14] Tumor-derived PGE_2 is believed to promote cancer progression by stimulating cell motility/invasion and tumor-associated angiogenesis and by preventing tumor cell apoptosis (reviewed in Cha and DuBois[15] and Wang and DuBois[16]). Several pathways and signaling networks are activated or enhanced by PGE_2;

Abbreviations used in this paper: CFSE, carboxyfluorescein succinimidyl ester; COX, cyclooxygenase; IFN, interferon; IL, interleukin; MLN, mesenteric lymph node; PGE_2, prostaglandin E2; IP, intraperitoneal; PAI, pathogenicity island; Th1, T-helper 1; Treg, regulatory T cell.

0016-5085/10/$36.00
doi:10.1053/j.gastro.2009.12.006

most notably, PGE_2 potentiates Wnt and peroxisome proliferator–activated receptor-δ signaling to induce target genes involved in replication and survival and triggers cell migration by activation of the epidermal growth factor receptor pathway.[15] In addition to its direct effects on cancer cell biology, PGE_2 is known to have strong immunosuppressive effects, giving rise to the hypothesis that PGE_2 production may allow tumors to evade immune surveillance.[17]

The reported immunosuppressive effects of PGE_2 on $CD4^+$ T cells, in particular T-helper 1 (Th1)-polarized effector T cells (reviewed in Harris and Phipps[18]), prompted us to examine the role of COX-2/PGE_2 in our model of *Helicobacter*-induced, T-cell–driven gastric preneoplasia. We found that inhibition of COX-2 enzymatic activity strikingly accelerated development of gastritis and premalignant gastric lesions and that, conversely, systemic administration of synthetic PGE_2 prevented premalignant changes. The protective effects of PGE_2 in our model could be attributed to its immunosuppressive effects on $CD4^+CD25^-$ T cells, which failed to migrate, to proliferate, and to secrete IFN-γ when exposed to PGE_2 in vitro or in vivo. Taken together, these data suggest that COX-2/PGE_2-dependent pathways are critical in preventing excessive gastric immunopathology by regulating the activity of "pathogenic" T cells.

Materials and Methods

Mice, Cell Culture, Bacterial Infections, and Vaccination

Male C57BL/6, interleukin (IL)-$10^{-/-}$BL/6, and Ly5.1BL/6 mice (Charles River Laboratories, Sulzfeld, Germany) were bred at a *Helicobacter*-free and specific pathogen-free facility and infected at 5 to 6 weeks of age. All animal experiments were approved by the cantonal veterinary office. *Helicobacter* strains used for animal experimentation were *H felis* CS1 (ATCC 49179)[19] and *H pylori* strain SS1.[20] Mouse infections were performed orally with $\sim 10^8$ bacteria. Primary gastric epithelial cells were isolated as described previously[21]; the immortalized murine gastric epithelial cell line used in this study was also described previously.[7] Both cell types were grown in Dulbecco's modified Eagle medium/Ham's F12 medium (1:1; Gibco, Basel, Switzerland) supplemented with 10% fetal calf serum and infected for 12 hours with *H pylori* strains G27 or an isogenic mutant lacking the Cag pathogenicity island (PAI), G27ΔPAI.[22] Vaccination of mice was performed as stated in the Supplementary Materials and Methods.

Pharmacological Treatments

For inhibition of COX-2, mice were fed a diet supplemented with 1500 ppm celecoxib (for details see Supplementary Materials and Methods; Research Diets, New Brunswick, NJ) or an identical control diet without the active compound. For PGE_2 treatment in vivo, 10 μg 16,16-dimethyl PGE_2 and 17-phenyl trinor PGE_2 were diluted in 100 μL phosphate-buffered saline (details in Supplementary Materials and Methods; Cayman Chemicals, Ann Arbor, MI) and injected twice intraperitoneally (IP) and once combined IP/orogastrically per week. IL-2 (BD Pharmingen, San Diego, CA) was injected IP 3 times per week (4×10^4 units per injection; see Supplementary Materials and Methods). For cell culture experiments, PGE_2 was added at 25 μM. IL-2 was used at 10 ng/mL.

Preparation of Gastric Tissue and Assessment of Colonization, Gastric IFN-γ Production, COX-2 Expression, Neutrophil Infiltration, and Histopathology

After sacrifice, stomachs were dissected longitudinally into equally sized pieces. For the assessment of *H felis* colonization, total genomic stomach DNA was subjected to quantitative polymerase chain reaction analysis of the *fla*B gene, as described previously.[7] Quantitative analysis of gastric IFN-γ expression and COX-2 expression and activity was performed as described in the Supplementary Materials and Methods. Neutrophil infiltration was quantified as described.[7] Paraffin sections were stained with the histological dyes Giemsa, Alcian Blue, and Periodic Acid Schiff, as well as an antibody specific for proliferating cell nuclear antigen (clone PC10; Zymed Labs, San Francisco, CA) for grading of gastric histopathology based on the features described in the updated Sydney classification (see Supplementary Materials and Methods).[23]

Lymphocyte Proliferation and Activation Assays

$CD4^+CD25^-$ T cells were immunomagnetically purified from single-cell suspensions of spleens, mesenteric lymph nodes, or stomach sections containing both corpus and antral tissue (R&D Systems, Minneapolis, MN). Cells were labeled with 5 μM carboxyfluorescein succinimidyl ester (CFSE) and stimulated with anti-CD3/CD28-coated beads. The CFSE dilution was assessed flow cytometrically. IFN-γ secretion was measured by enzyme-linked immunosorbent assay (BD Biosciences, San Diego, CA). IL-2 and IFN-γ expression was determined by reverse transcription polymerase chain reaction using primers specified in the Supplementary Materials and Methods. For staining of intracellular IFN-γ, cells were stained for CD4 (clone RM4-5; eBioscience, San Diego, CA) prior to fixation, permeabilization, and IFN-γ staining (XMG1.2, BD Biosciences). Fluorescence-activated cell sorting analyses were performed on a CyanADP instrument (Dako, Glostrup, Denmark). T-cell migration assay is described in the Supplementary Materials and Methods.

Statistical Analysis

P values were calculated using Graph Pad prism 5.0 software (GraphPad Software, San Diego, CA). The significance of differences in histopathology scores was

calculated by Mann–Whitney test and the significance of numerical differences was calculated by Student's *t* test. All in vitro assays were analyzed in triplicate and are shown with standard deviations.

Results

Helicobacter *Infection Induces COX-2 and PGE$_2$ In Vitro and In Vivo*

To assess whether COX-2 and its product PGE$_2$ are induced upon *Helicobacter* infection in cell culture models, we infected primary murine gastric epithelial cells (Figure 1*A*) and immortalized murine gastric epithelial cells (Figure 1*B*) with wild-type *H pylori* G27 or an isogenic mutant (ΔPAI) lacking the Cag pathogenicity island, a known virulence determinant of pathogenic *H pylori* strains.[22] In both cell types, COX-2 protein expression and PGE$_2$ secretion were induced by *H pylori* in a dose-dependent manner, and independently of the Cag PAI (Figure 1*A*–*C*). PGE$_2$ production was blocked in the presence of the COX inhibitor indomethacin (Figure 1*C*), indicating that it is produced exclusively by COX enzymes in this setting. COX-2 and its product PGE$_2$ were also strongly induced in the gastric mucosa of infected compared to uninfected mice (Figure 1*D* and *E*).

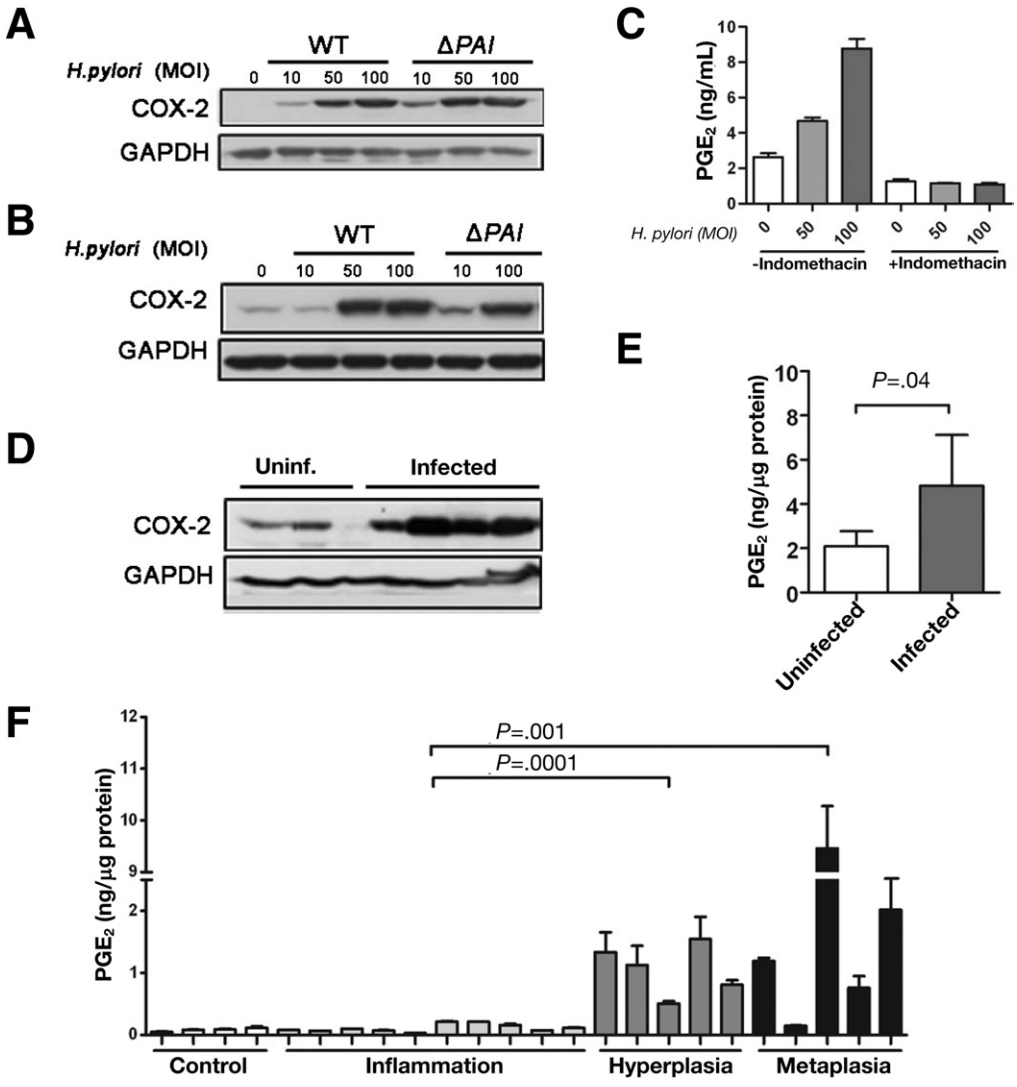


Figure 1. COX-2 protein expression and PGE$_2$ production is induced upon *Helicobacter* infection in vitro and in vivo. COX-2 protein expression was analyzed by Western blotting of extracts from primary murine gastric epithelial cells (*A*) and an immortalized murine gastric epithelial cell line (*B*) infected for 12 hours with either *H pylori* G27 wild-type (WT) or an isogenic Cag-PAI-deficient mutant (ΔPAI) at the indicated multiplicity of infections. Glyceraldehyde phosphate dehydrogenase (GAPDH) levels are shown as loading controls. PGE$_2$ production as assessed by competitive enzyme-linked immunosorbent assay (ELISA) is shown for supernatants of primary cells infected with WT G27, in the presence or absence of 28 μM indomethacin (*C*). For the analysis of COX-2 protein expression and PGE$_2$ production in vivo, whole stomach extracts were generated from four 5-month *H felis*–infected and 3 control C57BL/6 mice and subjected to Western blot analysis (*D*) and PGE$_2$ ELISA (*E*). (*F*) To determine mucosal PGE$_2$ concentrations as a function of pathology, we compared groups that showed gastritis only, hyperplasia, or metaplasia to uninfected controls. In (E) and (*F*), all samples were analyzed in triplicate; average values are shown with standard deviations.

In order to determine whether mucosal PGE_2 concentrations correlate with the degree and type of gastric pathology in our model, we selected groups of *Helicobacter*-infected mice based on their predominant pathology. We compared mice that showed gastritis, hyperplasia, or acidic mucus-positive metaplasia, respectively (for simplicity referred to as "metaplasia" in the following; Figure 1*F*). PGE_2 production was significantly higher in animals displaying epithelial damage, eg, hyperplasia and metaplasia, compared to those that showed gastritis only (Figure 1*F*). Our data are consistent with previous studies reporting COX-2 expression in *H pylori*–infected cultured epithelial cells[24] and in gastric preneoplastic and malignant lesions.[25]

COX-2 Inhibition During Infection Aggravates Helicobacter*-Induced Epithelial Pathology in C57BL/6 Mice*

To test if COX-2 activity modulates the formation of *Helicobacter*-associated chronic gastritis and precancerous lesions, we treated *H felis*–infected mice with celecoxib for 3 months (Figure 2*A*). Celecoxib specifically targets COX-2 and is believed to act predominantly by decreasing PGE_2 levels.[15] The efficacy of celecoxib treatment was confirmed by a COX-2–specific enzymatic activity assay (Figure 2*B*). The inhibition of COX-2 significantly aggravated *H felis*–induced epithelial atrophy and hyperplasia compared to infected, untreated counterparts as determined by quantitative histopathological scoring of histologically stained sections (Figure 2*C*). The more severe pathology observed under conditions of COX-2 inhibition was accompanied by a significant decrease in *H felis* colonization (Figure 2*D*), and a strong increase of the gastric expression of the Th1 cytokine IFN-γ (Figure 2*E*), corroborating both the direct correlation between IFN-γ levels and gastric histopathology, and the inverse correlation between IFN-γ expression and colonization that we have reported previously.[7] In conclusion, COX-2 inhibition accelerates development of *Helicobacter*-induced early gastric cancer precursor lesions, while simultaneously reducing the bacterial burden and increasing the local expression of IFN-γ, implying that COX-2 and PGE_2 may act as immune modulators of the *Helicobacter*–host interaction.

Treatment of Helicobacter*-Infected Mice With Synthetic PGE_2 Inhibits Chronic Inflammation and Precancerous Lesions*

To investigate a possible protective effect of COX-2 activity on the *H felis*–infected gastric mucosa in an independent experimental setup, we treated C57BL/6 mice with synthetic PGE_2 during 6 or 12 weeks of infection (Figure 3*A*). To ensure complete activation of all PGE_2 receptors (E-prostanoid receptors 1–4), we administered a mixture of 2 synthetic PGE_2 analogs, 16,16-dimethyl PGE_2 and 17-phenyl trinor PGE_2 (referred to in the following as "PGE_2"). The PGE_2 treatment prevented the mild to moderate lymphocytic infiltration observed after 6 weeks postinfection (Figure 3*A* and *B*) and also completely inhibited the moderate to severe chronic inflammation, atrophy, metaplasia, and/or hyperplasia indicative of 12-week–infected mice (Figure 3*A* and *C*). Immunohistochemical staining and quantification of proliferating cell nuclear antigen reactivity further confirmed the efficient inhibition of epithelial hyperproliferation by PGE_2 (Figure 3*D*). Consequently, the decrease in *H felis* colonization that typically accompanies the onset of severe gastric pathology between 6 and 12 weeks postinfection was prevented by PGE_2 treatment (Figure 3*E*). Long-term treatment with PGE_2 completely prevented the increase in local IFN-γ production that typically occurs in this time frame (Figure 3*F*) and that precedes the onset of pathology and leads to the observed reduction of the bacterial burden.[7]

We next tested the protective potential of PGE_2 in mice lacking the gene for the anti-inflammatory cytokine IL-10. IL-$10^{-/-}$ mice launch excessive proinflammatory and adaptive immune responses to *Helicobacter* infection;[26] as a result, they clear the bacteria and develop severe gastritis and preneoplastic lesions already 4 weeks postinfection (Figure 4). Treatment of infected IL-$10^{-/-}$ animals with synthetic PGE_2 efficiently inhibited development of gastric pathology and hyperproliferation (Figure 4*A*–***C***) and prevented neutrophil infiltration into the gastric mucosa (Figure 4*D*), but did not block clearance of the infection (data not shown). As in wild-type mice, local induction of IFN-γ was largely prevented by PGE_2 treatment in IL-$10^{-/-}$ mice (Figure 4*E*). The combined results indicate that prophylactic PGE_2 treatment efficiently inhibits development of chronic gastritis and cancer precursor lesions, possibly by modulating Th1-polarized T-cell responses.

Preexisting Gastric Cancer Precursor Lesions in H felis*–Infected Mice Can Be Reversed by PGE_2 Treatment*

To test the effects of PGE_2 as a potential therapeutic agent capable of curing preexisting lesions, we infected mice with *H felis* for 3 months and verified the efficient induction of preneoplastic pathology in a small control group (data now shown). Five of the remaining 13 mice received PGE_2 for another month (Figure 5*A*). Interestingly, this treatment efficiently reversed preexisting lesions, reducing the scores of all histopathological parameters (Figure 5*A* and *B*) and the fraction of corpus area affected by proliferating cell nuclear antigen–positive hyperplasia (Figure 5*C*), as well as neutrophil infiltration (Figure 5*D*). As in prophylactically treated mice, PGE_2 had a beneficial, in this case even curative, effect on mucosal integrity, while at the same time boosting bacterial colonization (Figure 5*E*). In line with all previous results (Figures 3 and 4), the PGE_2 treatment significantly reduced local gastric expression of IFN-γ (Figure 5*F*).

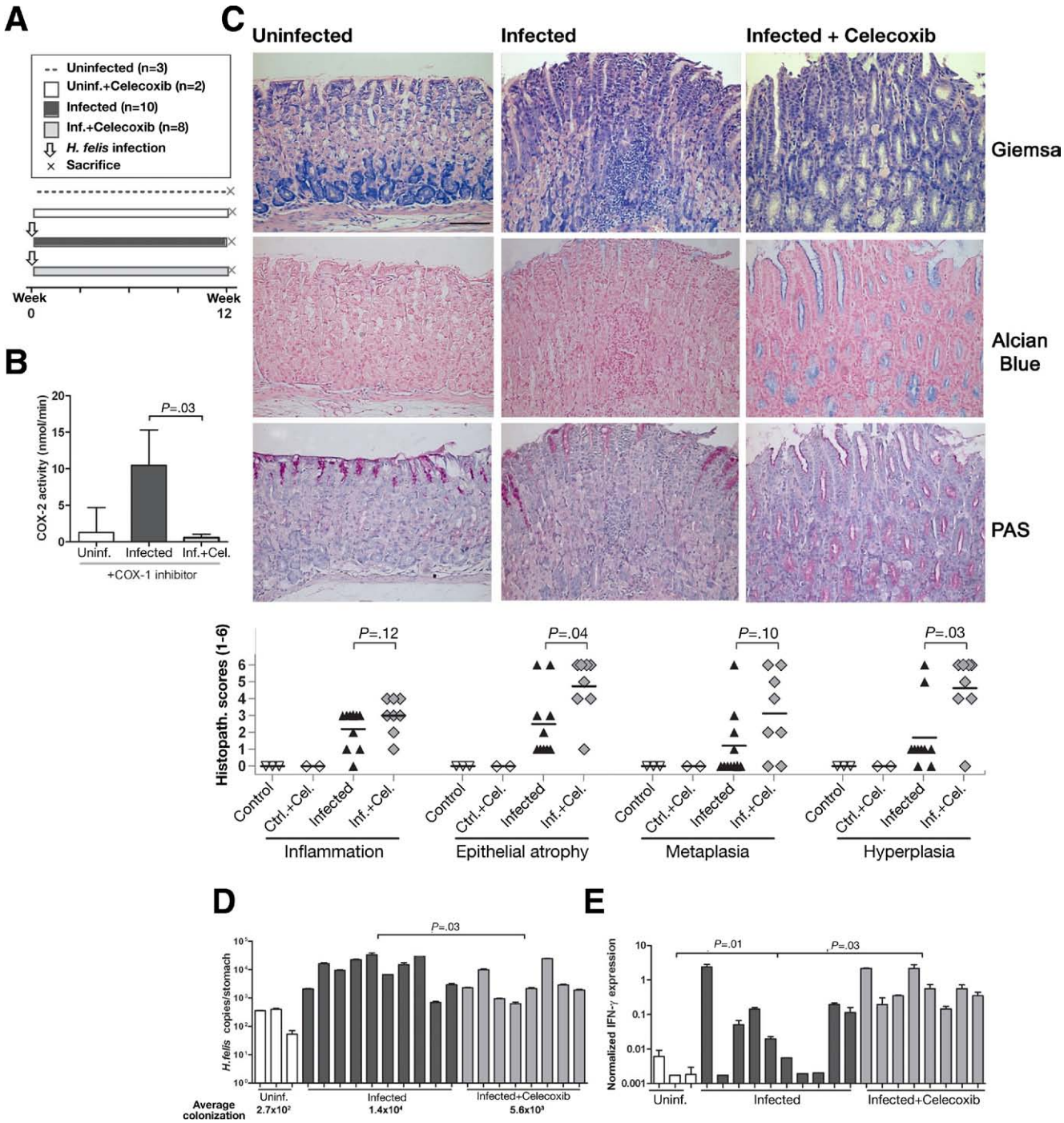


Figure 2. Inhibition of COX-2 by the diet-supplemented inhibitor celecoxib aggravates gastritis and gastric preneoplastic pathology induced by *H felis* infection. C57BL/6 mice were infected for 3 months with *H felis,* or remained uninfected, and were either fed a control diet or a diet supplemented with celecoxib (*A*). COX inhibition was verified by COX activity assay (*B*). All mice were analyzed with respect to gastric histopathology (representative Giemsa-, Alcian Blue-, and Periodic Acid Schiff–stained sections and pathology scores are shown. (*C*) *H felis* colonization (*D*) the average colonization is indicated per group and gastric expression of IFN-γ (*E*). Scale bar = 50 μm.

To confirm regression of preexisting lesions upon PGE$_2$ treatment during the last of 4 months of *H felis* infection, we repeated the experiment with 3 additional readouts (Supplementary Figure 1). As before, the lesions regressed in all PGE$_2$-treated mice compared to the infected control group (data not shown); we further found by flow cytometry that the gastric infiltration of leukocytes and CD4$^+$ T cells had significantly decreased in the PGE$_2$-treated group (Supplementary Figure 1*A* and *B*). Finally, we examined the presence of Th1-polarized, IFN-γ–producing–CD4$^+$ T cells in the mesenteric lymph nodes (MLN) by intracellular cytokine staining followed by fluorescence-ac-

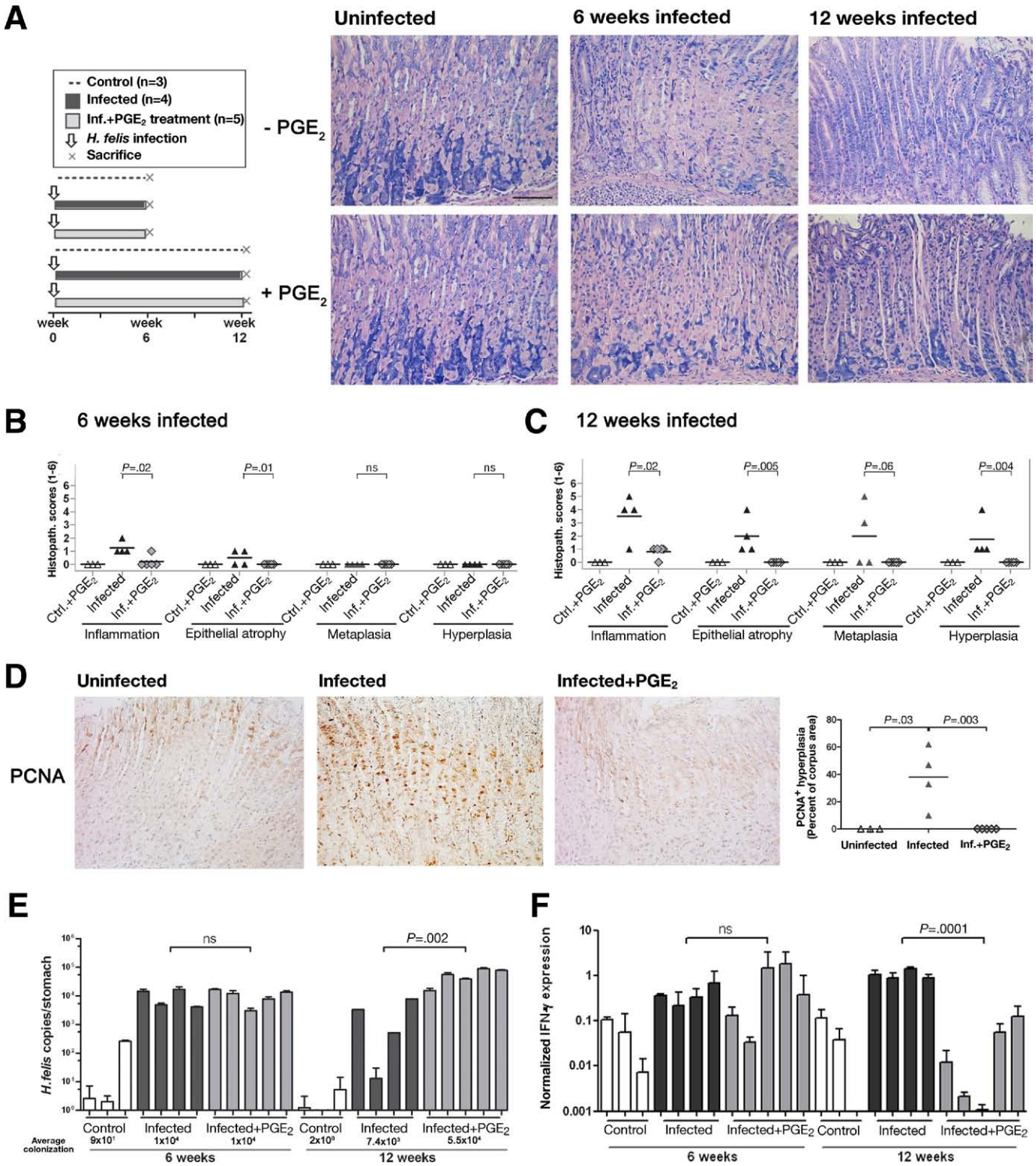


Figure 3. PGE_2 treatment of *H felis*–infected C57BL/6 wild-type mice inhibits chronic inflammation and epithelial pathology. C57BL/6 mice were infected with *H felis* and were treated with PGE_2 as indicated (*A*). Mice were euthanized 6 weeks (*A, B, E, F*) or 12 weeks postinfection (*A, C, E, F*). Representative Giemsa-stained sections and a summary of all histopathology scores are shown in (*A–C*); representative proliferating cell nuclear antigen (PCNA)–stained sections after 12 weeks of infection, along with their quantification, are shown in (*D*). *H felis* colonization and gastric IFN-γ expression are shown in (*E*) and (*F*).

tivated cell sorting. We found that the 4-month infection indeed led to a strong increase of IFN-γ–producing $CD4^+$ T cells in the MLN, which could be blocked at least partially by PGE_2 treatment (Supplementary Figure 1*C*). We conclude from both studies that systemically administered PGE_2 efficiently reverses preexisting gastric cancer precursor lesions, possibly by blocking the priming and activity of Th1-polarized T-cell responses in the draining lymph nodes.

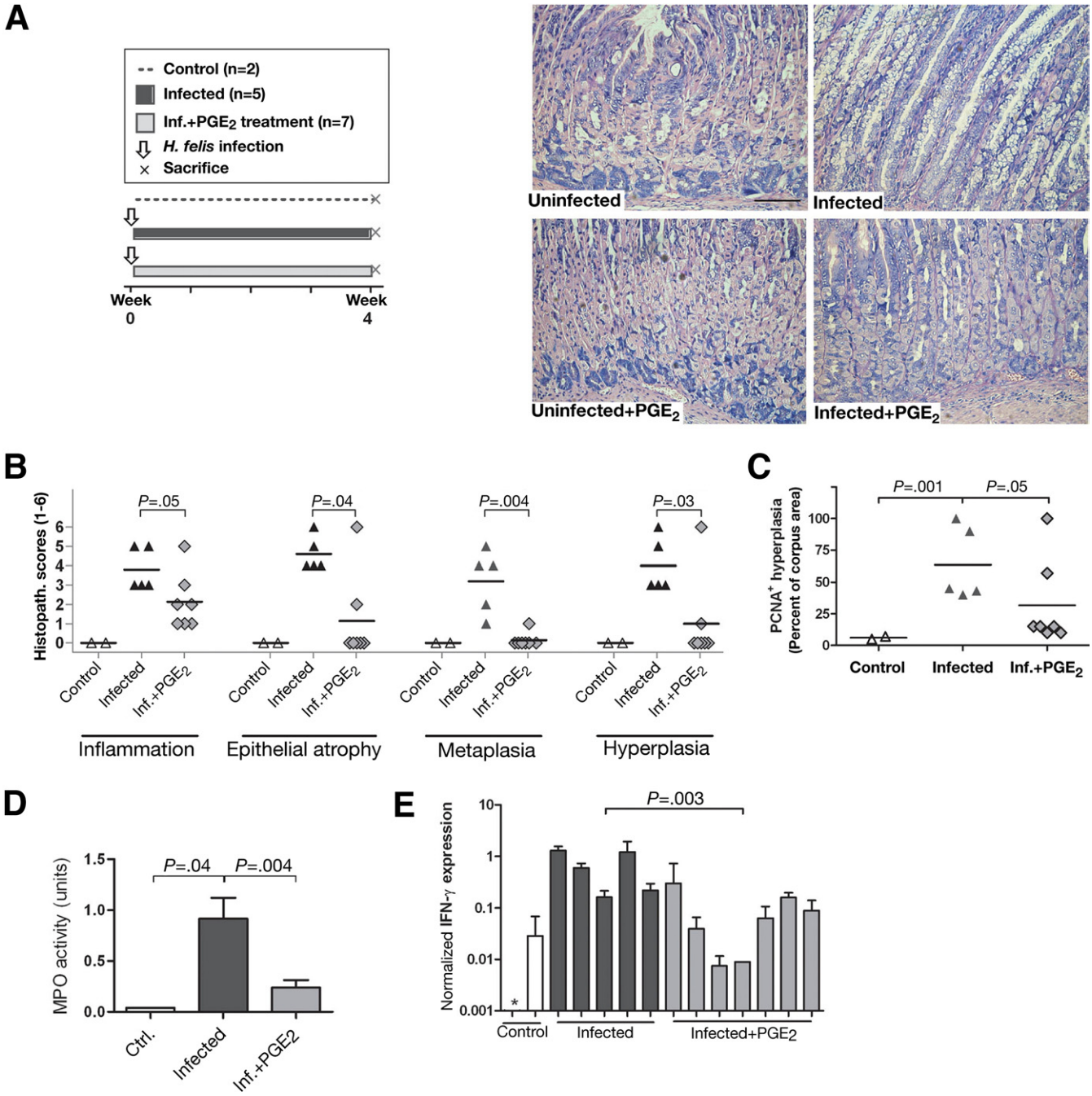


Figure 4. PGE$_2$ treatment of *H felis*–infected IL-10$^{-/-}$ BL/6 mice inhibits chronic inflammation and epithelial pathology IL-10$^{-/-}$ BL/6 mice were infected for 1 month with *H felis* and were treated with PGE$_2$ starting on the day of infection (*A*). Representative Giemsa-stained sections and histopathology scores are shown in (*A*) and (*B*); the quantification of proliferating cell nuclear antigen (PCNA)–positive hyperplasia is depicted in (*C*). Neutrophil infiltration was quantified by myeloperoxidase activity assay [averages of all mice per group are shown with standard deviations, (*D*)], and gastric IFN-γ expression was assessed by real-time polymerase chain reaction (*E*); (*not detectable).

PGE$_2$ Impairs Vaccine-Induced Protection Against Helicobacter *by Blocking Local Effector T-Cell Responses*

We have shown in the experiments outlined here that PGE$_2$ treatment in vivo boosts bacterial colonization and, conversely, that COX-2 inhibition promotes clearance (Figures 2*D*, 3*E*, and 5*E*). To test whether PGE$_2$ would also impair vaccine-induced protection upon challenge infection of immunized mice, we utilized a vaccination protocol directed against *H pylori* SS1. Indeed, administration of PGE$_2$ during challenge impaired the vaccine-induced reduction of bacterial loads (Figure 6*A*). The infiltration of leukocytes and CD4$^+$ T cells into the gastric mucosa observed in vaccinated/challenge-infected

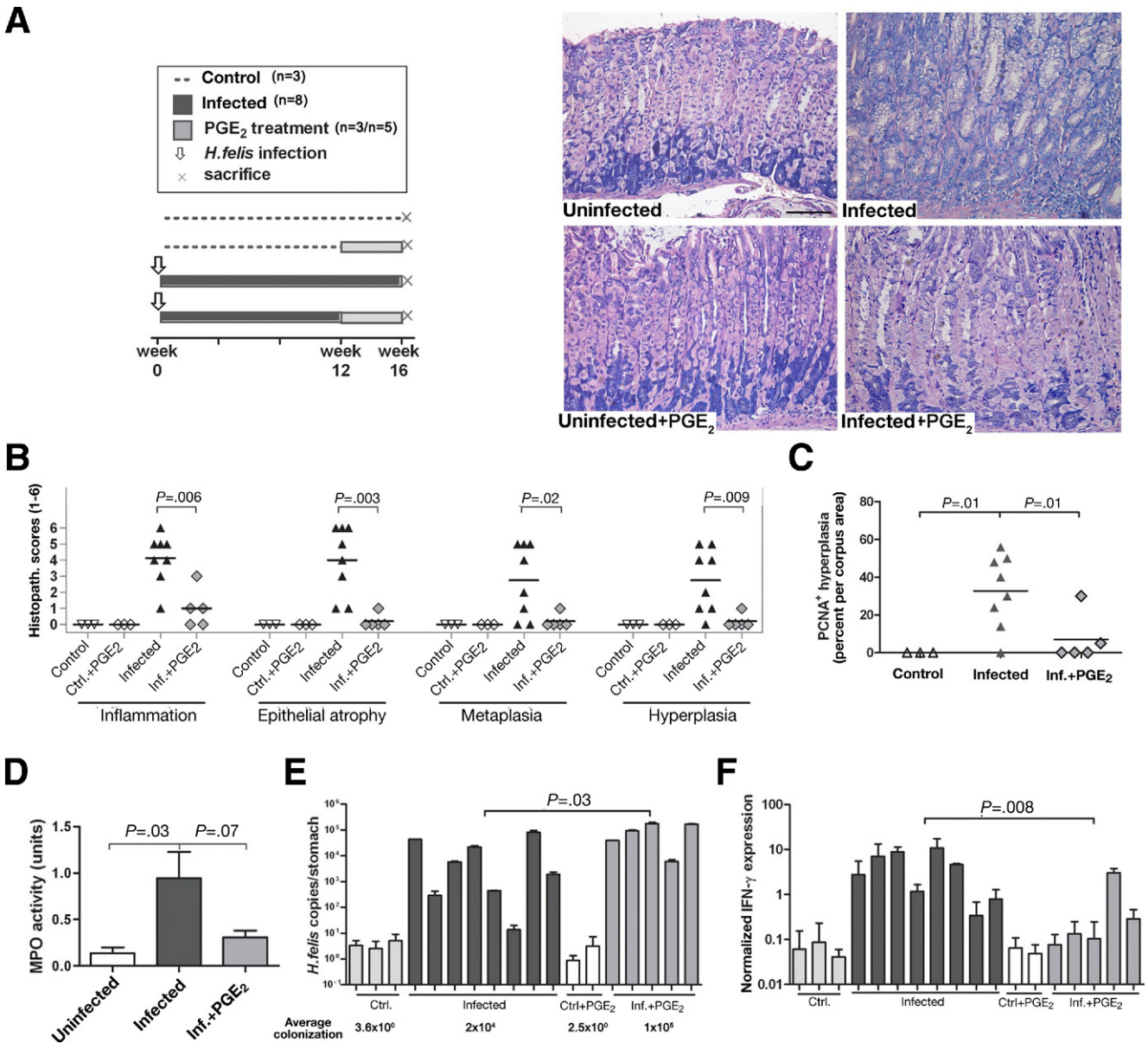


Figure 5. PGE_2 treatment of C57BL/6 mice reverses preexisting gastric precursor lesions. C57BL/6 mice were infected for a total of 16 weeks with *H felis* and treated with PGE_2 for the last month as indicated (*A*). Representative Giemsa-stained sections and histopathology scores are shown in (*A*) and (*B*); the quantification of proliferating cell nuclear antigen (PCNA)–positive hyperplasia in percent of the corpus area is depicted in (*C*). (*D*) Neutrophil infiltration was quantified by myeloperoxidase activity assay. *H felis* colonization and gastric interferon-γ expression are shown in (*E*) and (*F*).

mice was largely prevented by administration of PGE_2 (Figure 6*B* and *C*), possibly explaining why PGE_2-treated mice fail to reduce bacterial colonization. In single-cell MLN preparations of individual mice (Figure 6*D*), PGE_2 treatment during the challenge phase clearly abolished the vaccination-induced increase of IFN-γ–producing $CD4^+$ T cells in the MLN (Figure 6*D*). Taken together, our data imply that PGE_2 interferes with the T-cell–mediated control of *Helicobacter* infection, not only under standard experimental infection conditions, but also during a challenge infection after vaccination, and that this effect is due to T-cell inhibition in vivo.

To test whether PGE_2 directly interferes with IFN-γ production by MLN cells, single-cell suspensions were cultured in the presence of increasing concentrations of PGE_2 (Figure 6*E*). PGE_2 treatment reduced the infection-dependent secretion of IFN-γ into the supernatant by, on average, 43% and 94% at a low and high dose, respectively (Figure 6*E*). We conclude that PGE_2 efficiently blocks production of IFN-γ both in vitro and in vivo, which may explain why PGE_2-treated mice are protected from IFN-γ–induced preneoplastic pathology and show impaired bacterial clearance, a finding that is consistent with our previous report of an inverse link between colonization and pathology.[7]

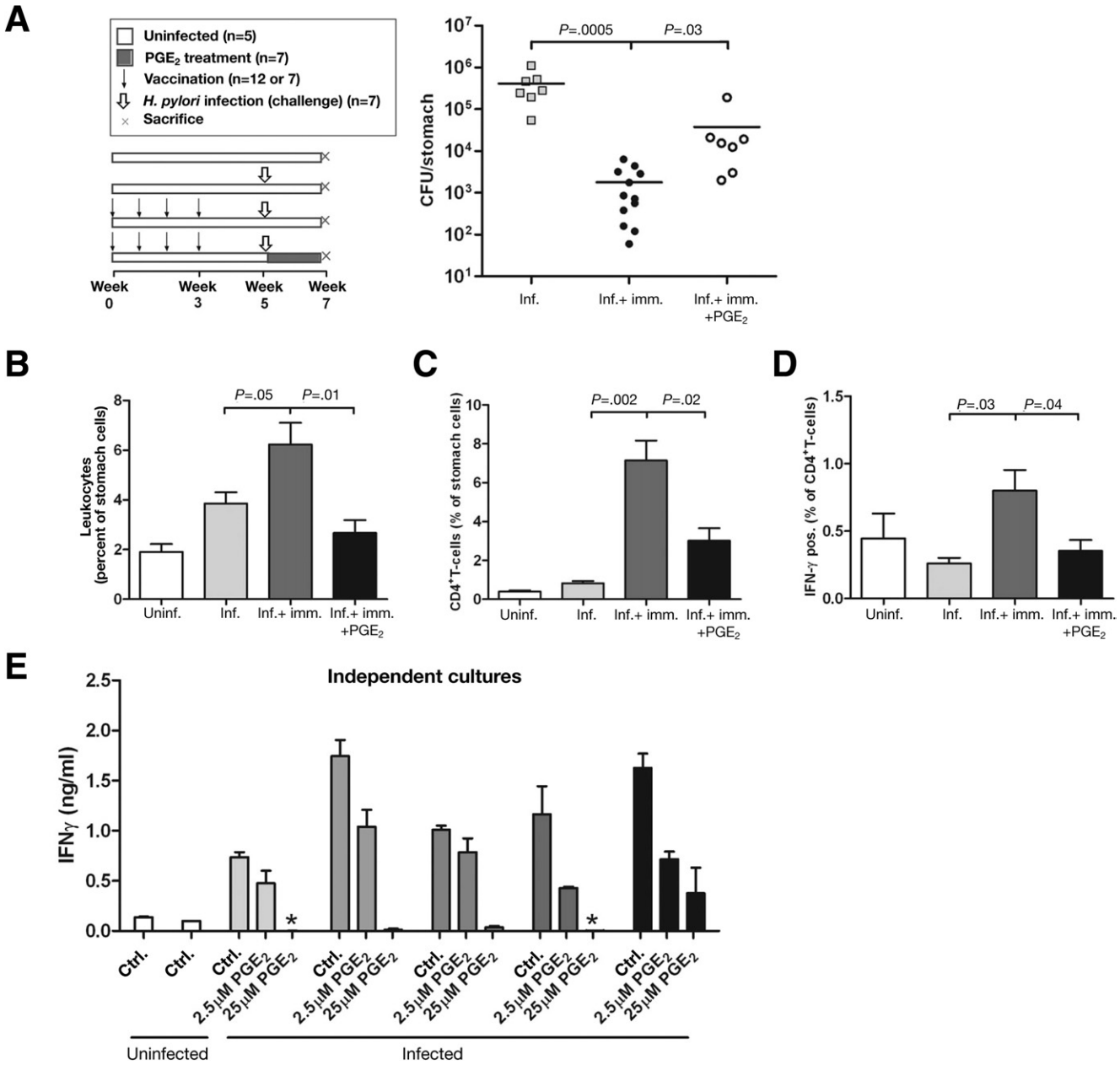
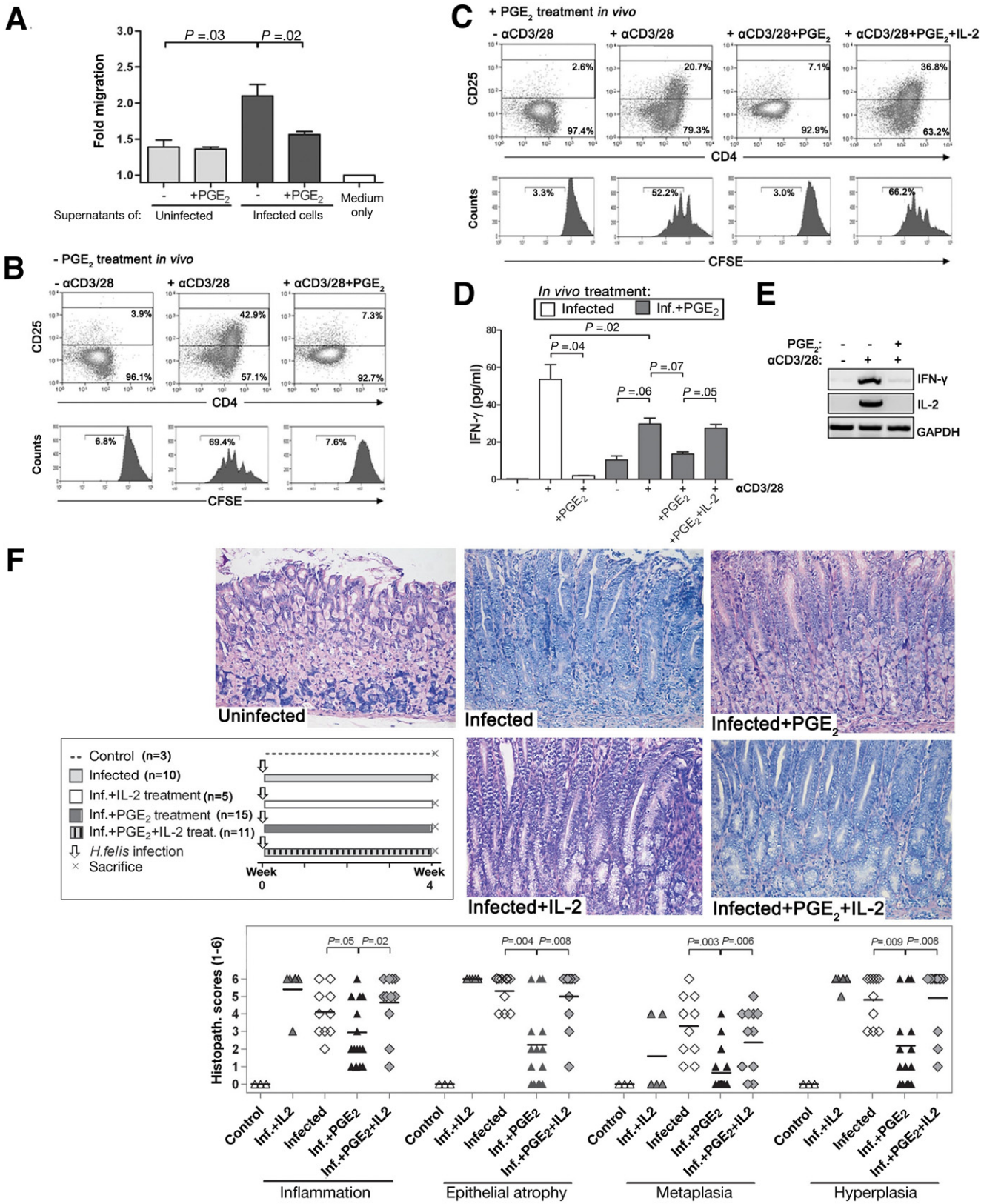


Figure 6. PGE$_2$ treatment during challenge infection impairs vaccine-induced clearance. C57BL/6 mice were vaccinated 4 times and challenged with live *H pylori* SS1; some mice received PGE$_2$ during the challenge phase as indicated. Nonvaccinated infected mice served as controls (*A*). Colony counts are shown as colony-forming unit (CFU)/stomach (*A*). (*B–D*) Single-cell suspensions from stomachs (*B, C*) and MLN (*D*) were generated for 2 uninfected, 4 control infected, 4 immunized/challenged, and 4 immunized/challenged/PGE$_2$-treated mice and analyzed individually by flow cytometry. Leukocyte (*B*) and CD4$^+$ T-cell (*C*) infiltration into the stomach is shown in percent of total stomach cells. (*D*) The IFN-γ–producing CD4$^+$ T-cell population in the MLN was quantified by intracellular cytokine staining. Averages and standard deviations are shown for every treatment group. (*E*) Single-cell MLN cultures obtained from 5 *H felis*–infected and 2 control mice were cultured overnight in the presence or absence of PGE$_2$ (2.5 μM or 25 μM). IFN-γ secretion into the supernatants was assessed by enzyme-linked immunosorbent assay (*not detectable).

PGE$_2$ Inhibits the Migration, Activation, and Effector Functions of Wild-Type CD4$^+$ T Cells

To test the effects of PGE$_2$ on CD4$^+$CD25$^-$ T-cell migration, we utilized a transwell migration assay in which calcein-labeled CD4$^+$CD25$^-$ T cells were allowed to migrate toward supernatants of *H pylori*–infected immortalized gastric epithelial cells. Interestingly, the supernatants of infected cells attracted T cells more efficiently than those of uninfected cells (Figure 7*A*). The migration could be blocked by addition of PGE$_2$ (Figure 7*A*). In order to assess the effects of PGE$_2$ on the proliferation and activation of CD4$^+$ effector T cells, we stimulated CFSE-labeled CD4$^+$CD25$^-$ T cells with anti-CD3/anti-CD28 monoclonal antibody–coated beads in the presence or absence of PGE$_2$ (Figure 7*B*). The activation


A
Fold migration
P =.03
P =.02
Supernatants of:
Uninfected
Infected cells
Medium only
B
- PGE2 treatment in vivo
- αCD3/28
+ αCD3/28
+ αCD3/28+PGE2
CD25
CD4
Counts
CFSE
C
+ PGE2 treatment in vivo
+ αCD3/28+PGE2+IL-2
D
In vivo treatment:
Infected
Inf.+PGE2
IFN-γ (pg/ml)
αCD3/28
E
PGE2:
αCD3/28:
IFN-γ
IL-2
GAPDH
F
Uninfected
Infected
Infected+PGE2
Infected+IL-2
Infected+PGE2+IL-2
Control (n=3)
Infected (n=10)
Inf.+IL-2 treatment (n=5)
Inf.+PGE2 treatment (n=15)
Inf.+PGE2+IL-2 treat. (n=11)
H.felis infection
Sacrifice
Week 0
Week 4
Histopath. scores (1-6)
Control
Inf.+IL2
Infected
Inf.+PGE2
Inf.+PGE2+IL2
Inflammation
Epithelial atrophy
Metaplasia
Hyperplasia

and proliferation of T cells, as assessed by measuring IL-2 receptor α chain (CD25) expression and CFSE dilution, was blocked almost completely by PGE_2 (Figure 7*B*). In vivo pretreatment of 5 donor mice with PGE_2 for 1 month prior to T-cell isolation also strongly reduced T-cell activation and proliferation upon CD3/CD28 cross-linking (compare Figure 7*B* and *C*). The secretion of IFN-γ by CD3/CD28-activated T cells was also blocked by PGE_2, both by the direct addition of the compound to the cultures and by pretreatment of the donor mice (Figure 7*D*). These results suggest that PGE_2 is an effective immunosuppressant capable of blocking the activation and proliferation of $CD4^+$ T cells, as well as their ability to secrete IFN-γ. $CD4^+$ effector T-cell activation and proliferation requires an autocrine-positive feedback loop of IL-2 secretion and IL-2 receptor activation.[27] We, therefore, asked whether the first step in this cascade, the transcriptional activation of the *IL-2* gene, could be blocked by PGE_2. Indeed, PGE_2 treatment efficiently inhibited *IL-2* and *IFNG* gene expression induced by CD3/CD28 cross-linking (Figure 7*E*). Therefore, we tested whether adding recombinant IL-2 would reverse the effects of PGE_2. Indeed, addition of IL-2 was able to completely reverse the effects of PGE_2 on T-cell activation, proliferation, and IFN-γ secretion (Figure 7*C* and *D*). These results suggest that PGE_2 inhibits T cells, at least in part, by interfering with their autocrine IL-2–driven positive feedback loop.

Based on studies reporting that PGE_2 may act as an immunosuppressant by stimulating the suppressive activities of regulatory T cells (Tregs),[28] we speculated that Tregs might be an additional target of PGE_2 in the control of T-cell–mediated immunopathology in the *Helicobacter*-infected stomach. To examine this possibility, we preconditioned immunomagnetically isolated $Ly5.2^+CD4^+CD25^+$ T cells with PGE_2 for 6 hours prior to coculturing them with CFSE-labeled $Ly5.1^+$ effector T cells at various ratios. Indeed, we found that PGE_2 pretreatment modestly enhanced the suppressive capacity of Tregs, albeit only at a ratio of suboptimal suppression (Supplementary Figure 2). This piece of data suggests that Tregs can indeed respond to PGE_2 in vitro and become more suppressive as a result of PGE_2 stimulation.

Recombinant IL-2 Reverses the Effects of PGE$_2$ In Vivo

In order to investigate whether the observed opposing effects of IL-2 and PGE_2 hold true in vivo, and to explore whether T cells are indeed critical targets of PGE_2, we utilized the previously mentioned $IL\text{-}10^{-/-}$ model (Figure 4). In addition to a group treated with PGE_2 only, we also included groups that received IP doses of recombinant IL-2 (4×10^4 units, 3 times weekly) together with PGE_2 or IL-2 alone (Figure 7*F*). IL-2 administration efficiently reversed the protection from gastric pathology conferred by PGE_2 (Figure 7*F*). This result suggests that the protective effect of PGE_2 on the infected gastric mucosa is due to the suppression of T-cell effector functions, which are known to directly trigger the atrophic gastritis, hyperplastic, and metaplastic transformation that are a hallmark of the infection in mice as well as in humans.[7]

Discussion

In this study, we provide experimental evidence that COX-2/PGE_2-dependent pathways play an important immunomodulating role during the early stages of *Helicobacter*-induced gastric pathogenesis, thereby preventing excessive gastric immunopathology. We show that systemic administration of PGE_2 prevented, and COX-2 inhibition exacerbated, *Helicobacter*-induced atrophic gastritis, epithelial hyperplasia, and metaplasia. These effects were due to the immunosuppressive effects of PGE_2 on T cells, which failed to proliferate and to secrete IL-2 and IFN-γ when exposed to PGE_2 in vitro or in vivo.

Our data are in line with several previous studies reporting exacerbated gastritis in *H pylori*–infected rodents under conditions of COX-1 or -2 inhibition or deletion of the *COX-1* or *-2* genes.[29–32] Similar effects were observed in human trials investigating the effects of COX inhibi-

BASIC–ALIMENTARY TRACT

Figure 7. PGE_2 treatment inhibits $CD4^+CD25^-$ T-effector cell migration, activation, proliferation, and IFN-γ secretion in vitro, and recombinant IL-2 reverses the effects of PGE_2 in vivo. (*A*) 1×10^5 Calcein-labeled splenic $CD4^+CD25^-$ T cells were seeded into transwell chambers and allowed to migrate toward supernatants of *H pylori*–infected or uninfected immortalized gastric epithelial cells in the presence or absence of 25 μM PGE_2. The migration was quantified spectrophotometrically; data are displayed with the baseline migration of cells toward unconditioned medium set as 1. (*B–E*), $CD4^+CD25^-$ cells were isolated from the combined MLN of 5 infected animals (*B,D,E*) or of 5 infected animals that had been pretreated with PGE_2 in vivo for 1 month (*C,D*). Cells were labeled with CFSE and stimulated with αCD3/28-coated beads; 25 μM PGE_2 and/or 10 ng/mL IL-2 was added where indicated. The CFSE dilution as a measure of proliferation (*lower panels* of *B,C*) and CD25 up-regulation as a measure of activation (*upper panels* of *B,C*) were assessed flow-cytometrically. All culture conditions were analyzed in triplicate; all statistical comparisons [$\pm$anti-CD3/CD28, $\pm PGE_2$, IL-2 in (*B*) and (*C*); + anti-CD3/CD28 with or without PGE_2 pretreatment in vivo] revealed significant differences ($P \leq .05$) for the readouts of CFSE dilution and of CD25 up-regulation. (*D*) The anti-CD3/CD28-induced secretion of IFN-γ into the culture supernatants was measured by enzyme-linked immunosorbent assay for the same cultures as shown in (*B*) and (*C*). (*E*) IL-2 and IFN-γ expression was assessed by semi-quantitative reverse transcription polymerase chain reaction. A representative experiment of 3 (*A*) and 4 (*B–E*) is shown. (*F*) $IL10^{-/-}$ BL/6 mice were infected for 1 month with *H felis* and treated with PGE_2 or recombinant IL-2 or a combination of the 2. Representative Giemsa-stained sections and histopathology scores are shown. No significant differences were observed between IL-2–treated groups $\pm$ PGE_2.

tion by aspirin in *H pylori*–infected, asymptomatic individuals, who suffered significantly more from mucosal injury and peptic ulcers than their uninfected counterparts.[33,34]

Despite the well-documented side effects of aspirin use on the gastric mucosa, the long-term, low-level use of aspirin or nonaspirin nonsteroidal anti-inflammatory drugs prevents development of gastric cancer; this association holds true even in a large meta-analysis including a total of 9 studies.[14] Similarly, treatment with the COX inhibitor, nimesulide, prevented a substantial portion of chemically induced gastric cancers in *H pylori*–infected mice.[35] The chemopreventive outcome of COX inhibition in studies examining cancer as the end point is generally believed to be due to the powerful effects of tumor-derived PGE_2 on cancer cell survival and invasion, tumor-associated angiogenesis, and on anti-tumor immunosuppression, all of which promote cancer progression.[15]

We demonstrate here that T cells, in particular effector subsets of the T-helper compartment, can be targeted by PGE_2. We have shown in a previous study that *Helicobacter*-induced gastric preneoplastic lesions result from diffuse infiltration of $CD4^+$, Th1-polarized effector T cells into the gastric lamina propria, where T-cell–derived IFN-γ acts directly on gastric epithelial lineages to promote their hyperproliferation and their transformation to intestinal-type cells.[7] Effector functions of $CD4^+$ T cells, such as proliferation, IFN-γ production, and migration are blocked upon exposure to PGE_2 in our models, with the underlying mechanism being a block of *IL-2* gene transcription. Consequently, administration of recombinant IL-2 was sufficient to override the protective effects of synthetic PGE_2 in vitro and in vivo. The profound effect of PGE_2 on $CD4^+$ T-cell effector functions, maintained even in explanted T cells restimulated ex vivo, also explains why PGE_2-treated mice are colonized at higher levels than untreated counterparts, and show impaired vaccine-induced protection. We conclude that PGE_2 averts immunopathology in the infected stomach by T-cell suppression, the necessary consequence of which is persistent colonization with *Helicobacter*. In seeming contrast to the protective effect of exogenously added PGE_2, we observe elevated endogenous PGE_2 in mice with established atrophy. We believe that this PGE_2, which may be COX-1–derived, is secreted by the gastric mucosa in an effort to repair the mucosal damage,[36] but is insufficient to act on T cells and to prevent further damage or to reverse preestablished lesions.

Our finding that PGE_2 treatment protects IL-$10^{-/-}$ mice from gastric pathology argues against Tregs being the primary target of PGE_2-driven immunosuppression as IL-$10^{-/-}$ Tregs are incapable of blocking the *Helicobacter*-associated gastritis induced by adoptive transfer of effector T cells into infected Rag-$2^{-/-}$ recipients.[37] However, we did observe that Tregs became modestly more suppressive in vitro upon pretreatment with PGE_2, as demonstrated previously by Sharma et al;[28] this observation suggests that the combined effects of PGE_2 on effector and regulatory T cells may together account for the complete block in T-cell–driven immunopathology observed in our wild-type infection models.

An intriguing finding of our study was the observed therapeutic effect of PGE_2 on preexisting lesions, an observation that is seemingly incongruent with studies reporting the prevention of gastric cancer by COX inhibition.[14,35] However, it has to be stressed that *Helicobacter*-associated preneoplastic lesions in our mouse models are benign and can be at least partially reversed upon antibiotic eradication of the infection.[38] Under these circumstances, the beneficial effect of PGE_2's suppression of pathogenic T cells apparently outweighs the potential pro-carcinogenic effects that PGE_2 may have on gastric epithelial cells themselves.[32]

In conclusion, our data are consistent with a protective role for PGE_2 during the early stages of the *Helicobacter*-induced gastric carcinogenesis sequence; at the molecular level, PGE_2 blocks the autocrine IL-2–driven positive feedback loop to prevent T-cell effector functions that are required for both *Helicobacter* clearance and induction of preneoplastic gastric immunopathology.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2009.12.006.

References

1. Parsonnet J, Friedman GD, Vandersteen DP, et al. *Helicobacter pylori* infection and the risk of gastric carcinoma. N Engl J Med 1991;325:1127–1131.
2. Parsonnet J, Isaacson PG. Bacterial infection and MALT lymphoma. N Engl J Med 2004;350:213–215.
3. Danesh J. *Helicobacter pylori* infection and gastric cancer: systematic review of the epidemiological studies. Aliment Pharmacol Ther 1999;13:851–856.
4. Fox JG, Sheppard BJ, Dangler CA, et al. Germ-line p53-targeted disruption inhibits *Helicobacter*-induced premalignant lesions and invasive gastric carcinoma through down-regulation of Th1 proinflammatory responses. Cancer Res 2002;62:696–702.
5. Rieder G, Merchant JL, Haas R. *Helicobacter pylori* cag-type IV secretion system facilitates corpus colonization to induce precancerous conditions in Mongolian gerbils. Gastroenterology 2005; 128:1229–1242.
6. Correa P. *Helicobacter pylori* and gastric carcinogenesis. Am J Surg Pathol 1995;19(Suppl 1):S37–S43.
7. Sayi A, Kohler E, Hitzler I, et al. The CD4+ T cell-mediated IFN-gamma response to *Helicobacter* infection is essential for clearance and determines gastric cancer risk. J Immunol 2009; 182:7085–7101.
8. Simmons DL, Botting RM, Hla T. Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. Pharmacol Rev 2004;56:387–437.
9. Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid receptors: structures, properties, and functions. Physiol Rev 1999;79:1193–1226.

10. Potter JD, Ulrich CM. COX-2 and gastric cancer: more on inflammation and neoplasia. Gastroenterology 2006;130:2198–2200.
11. Liu F, Pan K, Zhang X, et al. Genetic variants in cyclooxygenase-2: expression and risk of gastric cancer and its precursors in a Chinese population. Gastroenterology 2006;130:1975–1984.
12. Ulrich CM, Bigler J, Potter JD. Non-steroidal anti-inflammatory drugs for cancer prevention: promise, perils and pharmacogenetics. Nat Rev Cancer 2006;6:130–140.
13. Kune GA, Kune S, Watson LF. Colorectal cancer risk, chronic illnesses, operations, and medications: case control results from the Melbourne Colorectal Cancer Study. Cancer Res 1988;48: 4399–4404.
14. Wang WH, Huang JQ, Zheng GF, et al. Non-steroidal anti-inflammatory drug use and the risk of gastric cancer: a systematic review and meta-analysis. J Natl Cancer Inst 2003;95:1784–1791.
15. Cha YI, DuBois RN. NSAIDs and cancer prevention: targets downstream of COX-2. Annu Rev Med 2007;58:239–252.
16. Wang D, DuBois RN. Inflammatory mediators and nuclear receptor signaling in colorectal cancer. Cell Cycle 2007;6:682–685.
17. Pockaj BA, Basu GD, Pathangey LB, et al. Reduced T-cell and dendritic cell function is related to cyclooxygenase-2 overexpression and prostaglandin E2 secretion in patients with breast cancer. Ann Surg Oncol 2004;11:328–339.
18. Harris SG, Phipps RP. Prostaglandin D(2), its metabolite 15-d-PGJ(2), and peroxisome proliferator activated receptor-gamma agonists induce apoptosis in transformed, but not normal, human T lineage cells. Immunology 2002;105:23–34.
19. Lee A, Fox JG, Otto G, et al. A small animal model of human *Helicobacter pylori* active chronic gastritis. Gastroenterology 1990;99:1315–1323.
20. Lee A, O'Rourke J, De Ungria MC, et al. A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. Gastroenterology 1997;112:1386–1397.
21. Fujikawa A, Shirasaka D, Yamamoto S, et al. Mice deficient in protein tyrosine phosphatase receptor type Z are resistant to gastric ulcer induction by VacA of *Helicobacter pylori*. Nat Genet 2003;33:375–381.
22. Censini S, Lange C, Xiang Z, et al. cag, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. Proc Natl Acad Sci U S A 1996;93: 14648–14653.
23. Dixon MF, Genta RM, Yardley JH, et al. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. Am J Surg Pathol 1996;20:1161–1181.
24. Juttner S, Cramer T, Wessler S, et al. *Helicobacter pylori* stimulates host cyclooxygenase-2 gene transcription: critical importance of MEK/ERK-dependent activation of USF1/-2 and CREB transcription factors. Cell Microbiol 2003;5:821–834.
25. Sun JH, Das KK, Amenta PS, et al. Preferential expression of cyclooxygenase-2 in colonic-phenotype of gastric intestinal metaplasia: association with *Helicobacter pylori* and gastric carcinoma. J Clin Gastroenterol 2006;40:122–128.
26. Ismail HF, Fick P, Zhang J, et al. Depletion of neutrophils in IL-10(−/−) mice delays clearance of gastric *Helicobacter* infection and decreases the Th1 immune response to *Helicobacter*. J Immunol 2003;170:3782–3789.
27. Smith KA. Interleukin-2: inception, impact, and implications. Science 1988;240:1169–1176.
28. Sharma S, Yang SC, Zhu L, et al. Tumor cyclooxygenase-2/prostaglandin E2-dependent promotion of FOXP3 expression and CD4+ CD25+ T regulatory cell activities in lung cancer. Cancer Res 2005;65:5211–5220.
29. Li GQ, Xia HH, Chen MH, et al. Effects of cyclooxygenase-1 and -2 gene disruption on *Helicobacter pylori*-induced gastric inflammation. J Infect Dis 2006;193:1037–1046.
30. Takahashi S, Fujita T, Yamamoto A. Role of cyclooxygenase-2 in *Helicobacter pylori*-induced gastritis in Mongolian gerbils. Am J Physiol Gastrointest Liver Physiol 2000;279:G791–G798.
31. Tanigawa T, Watanabe T, Hamaguchi M, et al. Anti-inflammatory effect of two isoforms of COX in *H. pylori*-induced gastritis in mice: possible involvement of PGE2. Am J Physiol Gastrointest Liver Physiol 2004;286:G148–G156.
32. Lee CW, Rickman B, Rogers AB, et al. Combination of sulindac and antimicrobial eradication of *Helicobacter pylori* prevents progression of gastric cancer in hypergastrinemic INS-GAS mice. Cancer Res 2009;69:8166–8174.
33. Feldman M, Cryer B, Mallat D, et al. Role of *Helicobacter pylori* infection in gastroduodenal injury and gastric prostaglandin synthesis during long term/low dose aspirin therapy: a prospective placebo-controlled, double-blind randomized trial. Am J Gastroenterol 2001;96:1751–1757.
34. Wallace JL. How do NSAIDs cause ulcer disease? Baillieres Best Pract Res Clin Gastroenterol 2000;14:147–159.
35. Nam KT, Hahm KB, Oh SY, et al. The selective cyclooxygenase-2 inhibitor nimesulide prevents *Helicobacter pylori*-associated gastric cancer development in a mouse model. Clin Cancer Res 2004;10:8105–8113.
36. Starodub OT, Demitrack ES, Baumgartner HK, et al. Disruption of the Cox-1 gene slows repair of microscopic lesions in the mouse gastric epithelium. Am J Physiol Cell Physiol 2008;294:C223–C2232.
37. Lee CW, Rao VP, Rogers AB, et al. Wild-type and interleukin-10-deficient regulatory T cells reduce effector T-cell-mediated gastroduodenitis in Rag2−/− mice, but only wild-type regulatory T cells suppress *Helicobacter pylori* gastritis. Infect Immun 2007; 75:2699–2707.
38. Lee CW, Rickman B, Rogers AB, et al. *Helicobacter pylori* eradication prevents progression of gastric cancer in hypergastrinemic INS-GAS mice. Cancer Res 2008;68:3540–3548.

Received June 16, 2009. Accepted December 8, 2009.

Reprint requests

Address requests for reprints to: Anne Mueller, Institute of Molecular Cancer Research, University of Zürich, Winterthurerstr. 190, 8057 Zürich, Switzerland. e-mail: mueller@imcr.uzh.ch; fax: (41) 44 635 3484.

Acknowledgments

The authors wish to thank Esther Kohler for expert technical assistance. They are grateful to Dirk Bumann, Christoph Dehio, Michael Hottiger, Josef Jiricny, and Isabelle Arnold for helpful discussions. Gregor Fischer is thanked for advice with veterinary issues. The authors are grateful to Stanley Falkow for critical comments on the manuscript.

Conflicts of interest

The authors disclose no conflicts.

Funding

This study was funded by grants from the Swiss National Science foundation, the UBS foundation (BA29 S8Q7-DZZ 969/A), the Swiss Cancer League (OCS-02099-08-2007) and the Nils and Desiree Yde foundation to A.M. Additional funding was supplied by the University Research Priority Program in Systems Biology/Functional Genomics.

Supplementary Materials and Methods

Assessment of Gastric Histopathology

Paraffin sections were stained with the histological dyes Giemsa, Alcian Blue, and Periodic acid Schiff, as well as an antibody specific for proliferating cell nuclear antigen (clone PC10; Zymed Labs) for grading of gastric histopathology based on the features described in the updated Sydney classification.[1] We divided each of the 3 categories of the Sydney system (mild, moderate, and severe) into 2 subcategories as proposed by Chen et al,[2] resulting in a scale of 0–6 (none, 0; mild, 1–2; moderate, 3–4; severe, 5–6). The definitions of the 4 histopathological parameters scored were the following: chronic inflammation: infiltration of the mucosa and submucosa of the antrum and corpus of the stomach with B- and T-lymphocytes; atrophy: loss of specialized, terminally differentiated cells of the gastric mucosa, such as parietal, chief and mucus-producing cells (evident histologically as loss of surface staining with the Periodic Acid Schiff dye); hyperplasia: hyperproliferation of progenitor cells to compensate for the loss of differentiated gastric cells (therefore, sometimes called compensatory epithelial hyperplasia; detected by proliferating cell nuclear antigen reactivity); acidic mucus-producing metaplasia: appearance of acidic mucus-producing goblet-like cells in the gastric corpus mucosa, for simplicity reasons here referred to as metaplasia (histologically evident due to Alcian Blue positivity).

Quantification of COX-2 Expression and Enzymatic Activity, and PGE_2 Levels in Gastric Tissues

COX-2 expression was assessed by Western blotting (no. 160106; Cayman Chemicals, Ann Arbor, MI); COX-2 activity was determined using the COX activity Assay Kit (no. 760151; Cayman Chemicals) according to manufacturer's protocol with addition of COX-1 inhibitor. PGE_2 was assessed by competitive immunoassay (Assay Designs, Ann Arbor, MI) according to manufacturer's instructions.

Pharmacological Treatments (Extended Version)

For inhibition of COX-2, mice were fed a diet supplemented with 1500 ppm celecoxib (D04090202) as described by Jacoby et al[3] (Research Diets, New Brunswick, NJ) or an identical control diet (AIN 76A D1000) without the active compound. The average daily drug dose per mouse was determined to be 3.7 mg, based on the quantification of food intake per cage. For PGE_2 treatment in vivo and in vitro, a stock containing 1 mg/mL 16,16-dimethyl PGE_2 and 1 mg/mL 17-phenyl trinor PGE_2 was prepared in 100% ethanol, as described by Hansen–Petrik et al.[4] To this end, the methyl acetate in which PGE_2 is commercially available had to be vacuum-evaporated and then reconstituted in ethanol. The stock solution was diluted to 10 μg/100 μL in phosphate-buffered saline (PBS) and injected twice IP and once combined IP/orogastrically per week resulting in a total dose of 30 μg/week/mouse. Control mice were injected with PBS. For IL-2 treatment (BD Pharmingen; 50069, Lot 06498) in vivo, we administered 4×10^4 units per injection as proposed by Chong.[5] Recombinant IL-2 was diluted in PBS and administered 3 times weekly IP.

Vaccination of Mice, Challenge Infection, and Colony Count Assay

For vaccination, mice were gavaged 4 times at weekly intervals with 1 mg whole-cell sonicate of *H pylori* strain SS1 together with 10 μg cholera toxin as adjuvant (List Biologicals, Campbell, CA) prior to challenge infection with 10^8 colony-forming unit *H pylori* SS1. Mice were sacrificed 2 weeks postchallenge; bacterial colonization was assessed by plating of increasing volumes (1, 10, and 100 μL) of homogenized stomach extract on horse blood agar plates followed by colony counting after 5 days of culture under microaerophilic conditions as described previously.[6]

Determination of IL-2 and IFN-γ Expression by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and Real-Time RT-PCR

IL-2 and IFN-γ expression were analyzed by RT-PCR using the following primers: IL-2 forward: 5′GCGCACCCACTTCAAGCT 3′; IL-2 reverse: 5′CCTGCTTACAACACATAAGGC3′; IFN-γ forward: 5′GGTGACATGAAA ATCCTGCAGAGC3′; IFN-γ reverse: 5′ TCAGCAGCGACTCCTTTTCCGCTT 3′. The quantitative analysis of gastric IFN-γ expression was performed as described;[6] in brief, total RNA was isolated from scraped gastric mucosa using RNeasy Mini columns (Qiagen, Hilden, Germany). The corresponding complementary DNA served as template for real-time PCR (LightCycler; Roche, Mannheim, Germany) using the LightCycler 480 SYBR Green I master kit (Roche); glyceraldehyde phosphate dehydrogenase expression was determined for normalization.

Preparation of Protein Extracts for COX-2 and PGE_2 Quantification

Protein extracts of scraped gastric mucosa were prepared by homogenization in extraction buffer (20 mM HEPES, 0.4 mM NaCl, 25% glycerol, 1 mM EDTA, 1 mM NaF, and 0.1% NP-40, supplemented with protease inhibitors); 10 μg/mL indomethacin (a COX inhibitor; Sigma, St Louis, MO) was added to all extracts intended for PGE_2 quantification.

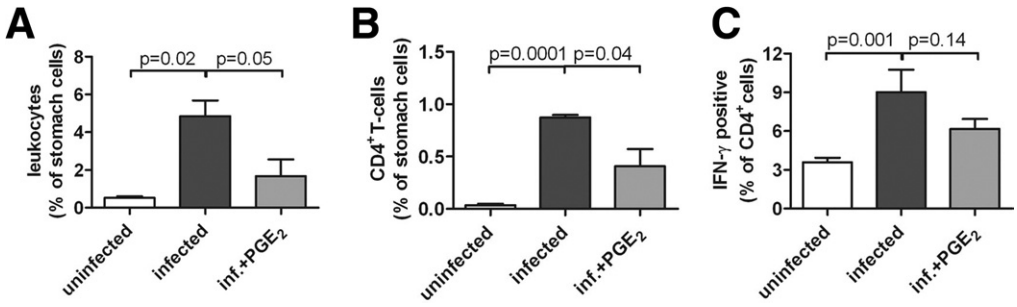
Transwell Migration Assay

$CD4^+CD25^-$ T-cells were generated as described here and labeled with calcein (BD Pharmingen); 10^5 lymphocytes were seeded onto 96-well format transwell

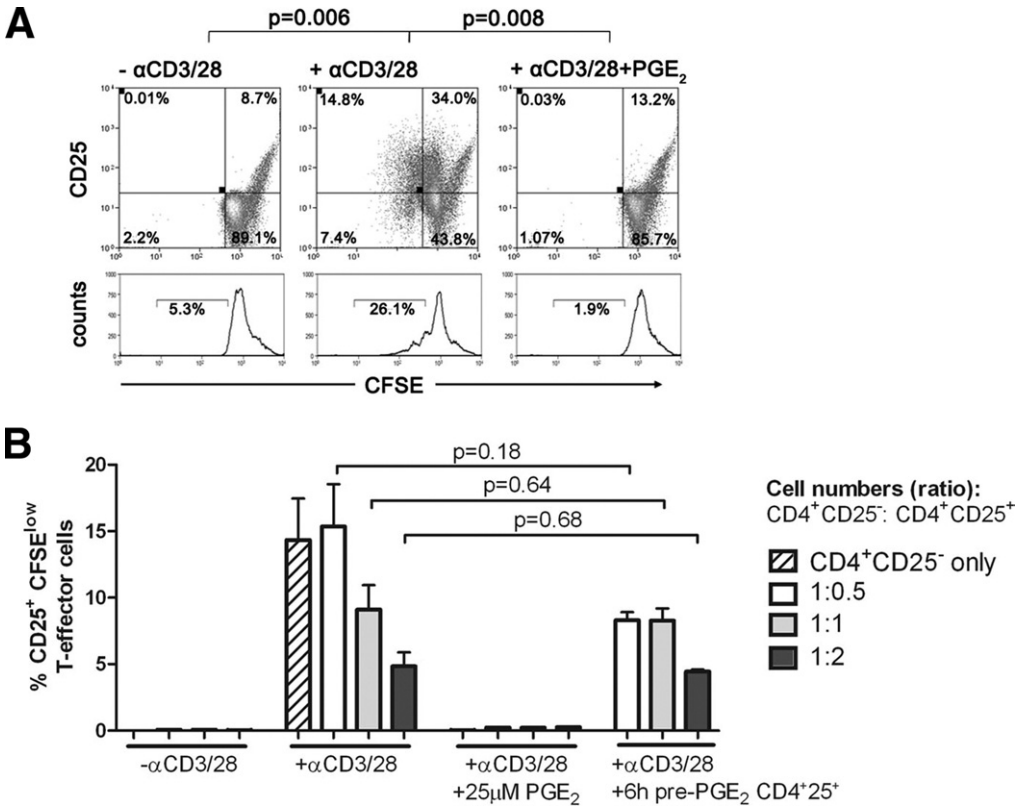
plates (ChemoTx Disposable Chemotaxis System, Neuro Probe, Gaithersburg, MD) and chemoattracted by 30 μL conditioned medium for 2 hours at 37°C. The proportion of migrated cells was quantified spectrophotometrically.

Supplementary References

1. Dixon MF, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. Am J Surg Pathol 1996;20:1161–1181.
2. Chen XY, van der Hulst RW, Bruno MJ, et al. Interobserver variation in the histopathological scoring of *Helicobacter pylori* related gastritis. J Clin Pathol 1999;52:612–615.
3. Jacoby RF, Seibert K, Cole CE, et al. The cyclooxygenase-2 inhibitor celecoxib is a potent preventive and therapeutic agent in the min mouse model of adenomatous polyposis. Cancer Res 2000;60: 5040–5044.
4. Hansen-Petrik MB, McEntee MF, Jull B, et al. Prostaglandin E(2) protects intestinal tumors from nonsteroidal anti-inflammatory drug-induced regression in Apc(Min/+) mice. Cancer Res 2002; 62:403–408.
5. Chong KT. Prophylactic administration of interleukin-2 protects mice from lethal challenge with gram-negative bacteria. Infect Immun 1987;55:668–673.
6. Sayi A, Kohler E, Hitzler I, et al. The CD4+ T cell-mediated IFN-gamma response to *Helicobacter* infection is essential for clearance and determines gastric cancer risk. J Immunol 2009; 182:7085–7101.



Supplementary Figure 1. Therapeutic PGE_2 treatment reduces the numbers of stomach-infiltrating leukocytes and $CD4^+$ T cells and blocks IFN-γ production in the MLN. C57BL/6 mice were infected for 3 months with *H felis* or remained uninfected (n = 5). A subgroup was treated with PGE_2 during the 4th month of infection (n = 5), whereas the remaining infected mice were left untreated (n = 5). Single-cell suspensions were generated from the stomach and MLN of every mouse and analyzed individually. The fraction of infiltrating leukocytes (A) and $CD4^+$ T-cells (B) in the stomachs and the fraction of $CD4^+$ IFN-γ^+ cells in the MLN (*C*) are shown. Averages and standard deviations were calculated per group.



Supplementary Figure 2. PGE_2 treatment moderately enhances the suppressive activity of $CD4^+CD25^+$ T cells on T-effector cell proliferation. $CD4^+CD25^-$ (Ly5.1BL/6) and $CD4^+CD25^+$(Ly5.2BL/6) T cells were immunomagnetically isolated from the MLN of uninfected mice. 1.5×10^5 CFSE–labeled $CD4^+CD25^-$ cells were stimulated with anti-CD3/CD28-coated beads in the presence or absence of 25 μM PGE_2 (*A*) or combined at increasing ratios with $CD4^+CD25^+$ Treg as indicated (*B*). PGE_2 was either added to combined cultures, or Treg were pretreated for 6 hours with PGE_2 and then added to $CD4^+CD25^-$ cells. Proliferation (CFSE dilution) and activation (CD25 expression) of T-effector cells were analyzed after 3 days by fluorescence-activated cell sorting. In (*B*), only the cells from the upper left quadrant (CD25+, CFSE low) are plotted. All cultures were analyzed in triplicate; averages and standard deviations are shown.